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(54) Title: IMMUNIZATION OF NON-HUMAN MAMMALS AGAINST STREPTOCOCCUS EQUI

(57) Abstract: The present invention is concerned with an antigenic composition comprising at least one antigen that comprises at least one antigenic epitope or antigenic determinant derived from a protein present in one or both of *S. equi* subsp. *Equi* and subsp. *Zooepidemicus* and use thereof for immunization of non-human mammals against *S. equi* subsp. *equi* and/or subsp. *Zooepidemicus*. The present invention also discloses a vaccine composition comprising the aforesaid antigenic composition as immunizing component. The antigens used are EAG, FNZ, SFS, SEC and ScLC.

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IMMUNIZATION OF NON-HUMAN MAMMALS AGAINST *STREPTOCOCCUS EQUI*

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**Background of the Invention****1. Field of the Invention**

This invention is generally related to antigenic compositions and use thereof for immunization of non-human mammals, e.g. horses, against *Streptococcus equi*.

10

Streptococcal infections in horses are mainly caused by the species *Streptococcus equi*, which is classified as a Lancefield Group C *Streptococcus* and comprises two subspecies designated *equi* and *zooepidemicus*, respectively.

15

*Streptococcus equi* subsp. *equi*, which is virtually confined to horses, is the causative agent of strangles, a world-wide distributed and highly contagious serious disease of the upper respiratory tract of the Equidae. Strangles is one of the most frequently reported equine diseases world-wide and is characterized by fever, nasal discharge, and abscess formation in the retropharyngeal and mandibular lymph nodes. In some cases the disease shows a metastatic course in the body, so called "bastard strangles". The disease has a world-wide distribution and causes great economic losses. Moreover, since strangles is a highly contagious disease, not only infected animals but also all other members of e.g. an afflicted stud must be isolated for as long as up to three months.

20

*S. equi* subsp. *zooepidemicus* is considered as an opportunistic commensal often occurring in the upper respiratory tract of healthy horses. However, after stress or virus infection, it can cause a secondary infection, which results in strangles-like symptoms. Moreover, subsp. *zooepidemicus* infects not only horses but also a wide range of other animals, like pigs, dogs, cats, and cows. Even human cases of infection due to subsp. *zooepidemicus* have been reported. This subspecies has been implicated as the primary pathogen in conditions such as endometritis, cervicitis, abortion, mastitis, pneumonia, abscesses and joint infections.

25

30

Although it is possible to treat and cure these streptococcal infections with antibiotics, such as penicillin, tetracycline or gentamicin, an effective prophylactic agent that could prevent outbursts of such infections and obviate or reduce the risk for development of resistant strains associated with antibiotic treatment, would be appreciated.

35

## 2. Description of the Related Art

However, although many attempts have been made to develop prophylactic agents such as vaccines against *S. equi*, at the present time no efficient vaccines or immunizing preparations are available, neither for the subspecies *equi* nor for the subspecies

5 *zooepidemicus*.

Existing vaccines against strangles are based on inactivated, e. g. heat-killed, or attenuated strains of *S. equi* subsp. *equi* or acid extracts/mutanolysin enriched in M-protein(s), i.e. immunogenic protein(s) produced by *S. equi*. A vaccine against *S. equi* subsp.

10 *zooepidemicus* based on an M-like protein is disclosed in US-A-5,583,014. In WO 87/00436, an avirulent strain of *S. equi* is disclosed for use as a vaccine against *S. equi* that stimulates an antibody response in the nasopharyngeal mucosa after administration thereof to a horse.

Since the previously developed vaccines or immunizing preparations are hampered by side-effects and, moreover, provide insufficient protection, there is a need for efficient and safe prophylactic agents, such as vaccines, that protect against *S. equi* infections and/or  
15 prevent spread thereof without giving rise to undesirable side-effects.

It is well known that attachment to eukaryotic cell surfaces is an essential step in the establishment of infection and colonization by bacterial pathogens. Accordingly, streptococcal surface proteins, that interact with and/or bind to different components of the Extracellular Matrix (ECM) or plasma proteins of the host cell, are potential candidates for use as active  
20 component(s) for immunizing purposes.

This is illustrated by the vaccines based on M-like proteins mentioned above or disclosed in the literature, i.a. in WO 98/0561. The binding of fibrinogen and complement factor H to M-proteins is assumed to be important for the ability of streptococci to resist phagocytosis by polymorphonuclear leucocytes.

25 Another mechanism used by streptococci for attachment to host cells involves binding to the ECM component fibronectin (Fn) (Ref. 21, 22). Binding between Fn-binding bacterial cell-surface proteins and immobilized Fn promotes internalization of streptococci by epithelial cells (Ref. 2, 23, 24). Fibronectin is a dimeric glycoprotein found both in plasma and in a fibrillar form in the extracellular matrix. The main function of Fn is to mediate  
30 substrate adhesion of eukaryotic cells, which involves the binding of specific cell-surface receptors to certain domains of the Fn molecule. Furthermore, it also interacts with several other macromolecules, such as DNA, heparin, fibrin, and collagen.

Accordingly, Fn-binding proteins from different streptococcal species have been cloned and sequenced previously. For instance, from *S. equi*, one Fn-binding protein has been

cloned and characterized, which is a Fn-binding cell-surface protein of subsp. *zooepidemicus*, that has been designated FNZ (Lindmark et al., 1996, Ref. 9). Another Fn-binding protein from *S. equi* subsp. *equi*, has been cloned and characterized by Lindmark and Guss (1999) (Ref. 12). This latter protein that is designated SFS and its potential use as an active  
5 component in a vaccine for protection of horses against strangles are disclosed in WO 00/37496.

In Jonsson et al. (1995) (Ref. 8), a protein designated ZAG has been cloned and characterized from *S. equi* subsp. *zooepidemicus* that mediates binding to the plasma proteinase inhibitor  $\alpha_2$ M. It is speculated therein that this protein is similar in function to  
10 streptococcal M proteins. This protein, ZAG, is also disclosed in WO 95/07296, where its  $\alpha_2$ M-binding properties are indicated. However, immunogenic properties or potential use thereof as an active component in a vaccine for protection of e.g. horses against strangles are not disclosed therein. The gene *zag* encoding ZAG is also disclosed in these references.

A gene that is similar to the aforesaid *zag* gene from *S. equi* subsp. *zooepidemicus*  
15 but is present in subsp. *equi* has been described by Lindmark et al. (1999) (Ref. 11) and Lindmark (1999) (Ref. 13). This gene is hereafter designated *eag* and encodes a protein designated EAG.

### Brief Summary of the Invention

The present invention is based on an antigenic composition comprising at least one  
20 antigen that comprises at least one antigenic epitope or antigenic determinant derived from a protein present in one or both of *S. equi* subsp. *equi* and subsp. *zooepidemicus* and use thereof for immunization of non-human mammals against *S. equi* subsp. *equi* and/or subsp. *zooepidemicus*.

The present invention is also directed to a vaccine composition comprising the afore-  
25 said antigenic composition as immunizing component, methods to prepare said antigenic or vaccine composition, methods to induce an immune response against *S. equi* in non-human mammals and methods for prophylactic or therapeutic treatment of *S. equi* infection in non-human mammals. When used generally, the expression "*S. equi*" refers to one or both of subsp. *equi* and subsp. *zooepidemicus*.

30 According to a suitable embodiment, the present invention is directed to a vaccine that protects equines, such as horses, against strangles.

In the context of infections caused by *S. equi* subsp. *equi*, the expression "non-human mammals" primarily refers to animals belonging to the family Equidae that consists of horses, donkeys and zebras and to hybrids thereof, such as mules and hinnies.

In connection with infections caused by *S. equi* subsp. *zooepidemicus*, the expression "non-human mammals" in addition refers also to other mammals such as cows, pigs, dogs and cats.

#### Brief Description of the Drawings

5 In the following, the present invention is described in closer detail with reference to the drawings, where

Fig. 1 shows IgG antibodies developed against FNZN in eight individual mice after intranasal inoculation with *Streptococcus equi* subsp. *equi*.

10 Fig. 2 shows IgG antibodies developed against SFSC1 in eight individual mice after intranasal inoculation with *Streptococcus equi* subsp. *equi*.

Fig. 3 shows IgG antibodies developed against EAG4B in eight individual mice after intranasal inoculation with *Streptococcus equi* subsp. *equi*.

15 Fig. 4 shows IgG antibodies developed in mice against FNZN after subcutaneous immunization with FNZN, SFSC1, and EAG4B. Results obtained for seven immunized mice and one non-immunized (▼) mouse are shown.

Fig. 5 shows IgG antibodies developed in mice against SFSC1 after subcutaneous immunization with FNZN, SFSC1, and EAG4B. Results obtained for seven immunized animals and one non-immunized animal (▼) are shown.

20 Fig. 6 shows IgG antibodies developed in mice against EAG4B after subcutaneous immunization with FNZN, SFSC1, and EAG4B. Results obtained for seven immunized animals and one non-immunized animal (▼) are shown.

25 Fig. 7 shows IgG antibodies developed in mice after subcutaneous immunization with SEC 2.16. Results from five immunized and five non-immunized mice are shown. (Pre-immune sera gave no response, values closest to the base line with symbols overlapping each other).

The results shown in Fig. 1-7, have been obtained with an ELISA test.

Fig. 8 shows the accumulated number of mice that survived or lost less than 15% of weight after subcutaneous immunization with FNZN, SFSC1, and EAG4B followed by challenge with *Streptococcus equi* subsp. *equi* (n= 24).

30 Fig. 9 shows nasal growth after subcutaneous immunization of mice with FNZN, SFSC1, and EAG4B followed by challenge with *Streptococcus equi* subsp. *equi*. Growth is determined on a scale of 0-3 (n= 24). Mean and SE (Standard Error) are shown. The control group was not immunized.

**Fig. 10** shows weight loss of mice after intranasal immunization of mice with FNZN, SFSC1, and EAG4B followed by challenge with *Streptococcus equi* subsp. *equi* (n= 24). The control group was not immunized.

**Fig. 11** shows nasal growth after intranasal immunization of mice with FNZN, SFSC1, and EAG4B followed by challenge with *Streptococcus equi* subsp. *equi* (n= 24). Mean and SE are shown.

**Fig. 12** shows IgA antibodies against SFSC1 in bronchoalveolar lavage (BAL) and nasal washes (NW) from mice nasally immunized with FNZN, SFSC1, and EAG4B. Results from six animals are shown.

**Fig. 13** shows weight loss of mice after intranasal immunization of mice with EAG4B followed by challenge with *Streptococcus equi* subsp. *equi* (n=10). The control group was not immunized.

**Fig. 14** shows IgG antibodies in immunized horses developed against EAG4B and FNZN.

**Fig. 15** shows the results from a comparison of serum antibody titers from horses with or without a history of strangles.

**Fig. 16** shows the presence of IgA antibodies in nasal washings from immunized horses.

**Fig. 17** shows antibody titers against EAG4B in horses immunized with EAG4B, FNZN, and SFSC1 as a function of time

**Fig. 18** shows the results obtained for mice (n=15) that were immunized with SEC2.16 using Matrix as adjuvant. The number of animals that lost more than 15% of weight or that died is shown as a function of time.

**Fig. 19** shows the results obtained for mice that were immunized with SEC2.16 alone (group B (n=5)) or SEC2.16 together with Matrix (group A (n=5)). Serum samples were taken before (denoted pre) and after (denoted imm) immunization.

### Detailed Description of the Invention

The present invention is directed to an antigenic composition comprising at least one antigen, wherein said at least one antigen comprises at least part of a protein of *Streptococcus equi* subsp. *equi*, and said at least part of said protein comprises at least one antigenic epitope or antigenic determinant of *Streptococcus equi*, and wherein said protein is comprised of a

protein that is designated EAG and comprises an N-terminal amino acid sequence, which is shown below as SEQ. ID. NO: 1 and is designated EAG4B.

SEQ. ID. NO: 1:

1 MALDATTVLE PTTAFIREAV REINQLSDDY ADNQELQAVL ANAGVEALAA DTVDQAKAAL  
5 61 DKAKAAVAGV QLDEARREAY RTINALSDQH KSDQKVQLAL VAAAAKVADA ASVDQVNAAI  
121 NDAHTAIADI TGAALLEAKE AAINELKQYG ISDYVVTLIN KAKTVEGVNA LKAKILSALP

According to a suitable embodiment of the present invention, said antigenic composition comprises at least one further antigen that comprises at least part of a fibronectin-binding protein of *Streptococcus equi*, said at least part of said protein comprising at least one  
10 antigenic epitope or antigenic determinant of *Streptococcus equi*, and wherein said protein is selected from the group consisting of FNZ (EMBL sequence data bank accession number X99995) comprising an amino acid sequence as shown in SEQ. ID. NO: 2 below:

MKTKSFRKVLTTSATCIVLATSFAGGTLRVWAEQLYYGWNDGTRQSSPYFLYVSPKNAPKRE  
LKDEYVVYCFNKKLYWPDQWESIYSNFNDIRSPYNDLPVYEKKLGYDGI FKQYAPDYKKDIS  
DIASALVAVLSNGYPTNKSQSLTSYHLNNDSSRKVTQLAIWYFSDSLTKEYLKDTGGYNLND  
MEKKALDFLISKGEDSKLKSEQSNYSLDIYVYQSGGHDHMKDYQNLGSLTIPEPLKPKQLG  
GFSGHNGNGLSGLEGGSSGSQETNEDGKKGLIGFHGGLSGSEGKRDPLPGLKGEAGAPDTPQ  
KPNDPLOGLEGGNSPIVEQNYGSTEGYHGQSGILEETEDTNPPGIILGGSGNVETHEDTRNP  
HLMGIGGGLAGESGETTPKPGQTGGQGPVIETTEDTQKMSGQSGGTIESENTKKPEVMIGG  
QGQTIETTEDTQKMSGQSGGTIESEDTKKPEVMIGGQGQIIDFSENTQSGMSGQSGDTTVI  
EDTKKSEIIIGGQGQIIDFSEDTPGMSGQSGGTIIVEDTKKPTPKPKPAPAPIVNDEKPNK  
GTHLPQTSMDKQLTSLIIGAMSMMLLVCLSLFKRPSKKD

and SFS comprising an amino acid sequence as shown in SEQ. ID. NO: 3 below:

15 MRKTEGRFRTWKSKKQWLFAGAVVTSLLLGAALVFGGLLGSLGG  
SSHQARPKEQPVSSIGDDDKSHKSSSDSMVSRPPKKNLQPKPSDQPTNHQHQAATSPS  
QPTAKSSGHHGNQPQSLSVNSQGNSSGQASEPQAIPNQGPSQPLGLRGGNSSGSGHHH  
20 QPQGKPPQHLDLGKDNSSPQPQPKPQGNSPKLPEKGLNGENQKEPEQGERGEAGPPLSG  
LSGNNQGRPSLPGLNGENQKEPEQGERGEAGPPSTPNLEGNNRKNPLKGLDGENKPKE  
25 DLDGKGLSGENDESPKLDDEHPYNHGRRDGYRVGYEDGYGGKKHKGDYPKRFEDESSPK  
EYNDYSQGYNDNYGNGYLDGLADRGGKRGYGYSYNPD.

Suitably, the afore-mentioned antigenic compositions also comprise at least one further antigen that comprises at least part of an extracellular matrix-binding protein of *Streptococcus equi* and said at least part of said protein comprises an antigenic epitope or an  
30 antigenic determinant of *Streptococcus equi* and wherein said protein is comprised of a

protein that is designated SEC and comprises an amino acid sequence as shown in SEQ. ID.

NO: 4 below.

SEQ. ID. NO: 4:

1  
27  
5 LKQLTKIVSVVLLL VFTLSASLHKVRATNLSDNITSLTVASSSLRDGERTTVKVAFDD  
KKQKIKAGDTIEVTWPTSGNVYIQGFNKTIPLNIRGVDVGTLEVTLDKAVFTFNQNE  
TMHDVSGWGEFDITVRNVTQTTAETSGTTTVKVGNRATITVTKEAGTGTSSFYK  
TGDMQPNDTERVRWFLINNKEWVANVTVEDDIQGGQTLDMSSFDITVSGYRNE  
RFVGENALTEFHTTTPNSVITATDNHISVRLDQYDASQNTVNIA YKTKITDFDQKEFA  
10 NNSKIWYQILYKDVSGQESNHQVANINANGGVDGSRYSFTVKKIWN DKENQDGK  
RPKTITVQLYANDQKVNDKTIELSDTNSWQASFGKLDKYDSQNQKITYSVKEVMVP  
VGYSQVEGDSGVGFTITNTYTPEVISITGQKTWDDRENQDGKRPKEITVRLANDA  
ATDKVATASEQTGWKYTFTNLPKYKD GKQITYTIQEDPVADYTTTIQGF DITNHHEV  
ALTSLKVIKVWNDKDDYYHKRPKEITILLKADGKVIREHQMTDPDQ QGKWEYTFDQL  
15 PVYQTGKKISYSIEEKQVAGYQAPVYEVDGLKQVTVTNTLNPSYKLPDTGGQGVK  
WYLLIGGGFIIVAILVLISLYQKHKRHNMSKP

657

The antigens of the present antigenic compositions may comprise the entire amino acid sequence of said protein or may comprise a fragment or analog thereof. Suitable  
20 fragments are N-terminal fragments of EAG and FNZ. An antigen derived from SFS may be comprised of a C-terminal part of SFS and an antigen derived from SEC may be comprised of a collagen-binding part of SEC.

A preferred antigenic composition of the present invention contains all the afore-said antigens EAG, FNZ, SFS, and SEC. Preferably, an N-terminal part of each of EAG and FNZ  
25 designated FNZN and EAG4B, respectively, a C-terminal part of SFS designated SFSC1 and a collagen-binding part of SEC designated SEC 2.16 are used in said composition.

From the above, it is evident that the present antigens that are derived from proteins of *Streptococcus equi* may comprise the entire protein, a fragment of said protein or an analog of said protein. Thus, the present invention is not limited to the fragments of proteins that are  
30 specifically disclosed herein.

A further embodiment of the present invention is concerned with a vaccine composition for protecting non-human mammals against infection of *Streptococcus equi*, which comprises an antigenic composition as disclosed above as immunizing component, and a pharmaceutically acceptable carrier.

35 Suitably, the present vaccine composition comprises an antigenic composition that contains all the afore-said antigens as immunizing component. Optionally, one or more of these antigens are comprised of analogs of said proteins or fragments thereof, e.g. N-terminal or C-terminal fragments.



The vaccine composition may comprise further components, such as an adjuvant. Suitably, said adjuvant stimulates systemic or mucosal immunity. Such adjuvants are well known in the art.

According to a suitable embodiment, the vaccine composition is a vaccine that  
5 protects susceptible mammals, suitably horses, against strangles caused by *Streptococcus equi* subsp. *equi*.

The vaccine composition of the present invention is provided in a physiologically administrable form. Suitably, it is administrable by subcutaneous or intranasal inoculation. Suitably, the vaccine composition of the present invention stimulates serum, mucosal and/or  
10 bronchial lavage antibody responses directed to *Streptococcus equi* antigens in mammals susceptible to *Streptococcus equi*, suitably horses.

The present invention is also related to a method for producing an antigen to be used in an antigenic composition of the present invention, which method comprises

- (a) providing a DNA fragment encoding said antigen and introducing said fragment  
15 into an expression vector;
- (b) introducing said vector, which contains said DNA fragment, into a compatible host cell;
- (c) culturing said host cell provided in step (b) under conditions required for expression of the product encoded by said DNA fragment; and
- 20 (d) isolating the expressed product from the cultured host cell.

Preferably, said method further comprises a step (e) wherein the isolated product from step (d) is purified, e.g. by affinity chromatography or other chromatographic methods known in the art.

A further embodiment of the present invention is concerned with a method for  
25 preparation of a vaccine of the present invention, which vaccine contains as immunizing component an antigenic composition as disclosed above, said method comprising mixing said antigenic composition and a pharmaceutically acceptable carrier.

The present invention is also related to a method for the production of an antiserum, said method comprising administering an antigenic preparation of the present invention to an  
30 animal host to produce antibodies in said animal host and recovering antiserum containing said antibodies produced in said animal host.

Moreover, the present invention is concerned with a method of prophylactic or therapeutic treatment of *S. equi* infection in non-human mammals, suitably horses, comprising

administering to said mammal an immunologically effective amount of a vaccine or an antiserum of the present invention.

A suitable embodiment of the present invention is concerned with a method for protecting horses against *Streptococcus equi* infection, which comprises inoculating a horse subcutaneously or intranasally or both subcutaneously and intranasally with a vaccine of the present invention to induce an immune response against *Streptococcus equi* in said horse. Suitably, an immune response in the form of IgG and IgA antibodies in the nasopharyngeal mucus is thereby induced in said horse.

The present invention describes the composition of a vaccine comprising one or several antigen components which have been prepared according to the present method using *E. coli* as host cells. The source of these antigens might also be the native bacteria, if methods are developed for expression and purification thereof. Alternatively, the antigens of the present invention can also be produced according to methods that are based on fusion strategies where various parts of the respective antigen are recombined resulting in a fusion protein consisting of parts from different antigens. This fusion strategy could also be suitable for introducing immune reactive part(s), e.g. T-cell epitopes or attenuated toxins (or parts thereof), thereby introducing other features suitable for optimizing the antigen presentation or localization.

## EXPERIMENTAL PART

### Example 1. Preparation of antigens derived from EAG

A gene similar to the afore-mentioned *zag* gene (the GeneBank accession number for the nucleotide sequence of *zag* is U2582) from *S. equi* subsp. *zooepidemicus* but present in subsp. *equi* has been described in Lindmark et al (1999) (Ref. 11) and Lindmark (1999) (Ref. 13). This gene that is present in subsp. *equi* and encodes the afore-mentioned protein EAG is hereafter called *eag*. To clone and express a part of this gene encoding an N-terminal fragment (EAG4B) of EAG in *E. coli*, the following work was done.

#### Construction

The primers

OZAG43B: 5'-TTT TCT CGA GCT ACG GTA GAG CTG ATA AAA TCT C-3'

(SEQ. ID. NO: 5) and

OZAG15: 5'-TCA GCC ATG GCT CTA GAT GCT ACA ACG GTG TT-3'

(SEQ. ID. NO: 6)

were used to PCR-amplify a DNA-fragment corresponding to amino acid residues 34-262 in protein EAG using *S. equi* subspecies *equi* 1866 chromosomal DNA as a template. The PCR-

product was digested with *Nco*I and *Xho*I and ligated into the pTYB4-vector obtained from New England Laboratories (NEB Inc) digested with the same enzymes. One µl of the ligation mixture was transformed into *E. coli* ER2566. Correct transformants were identified by colony screening using horse radish peroxidase-labeled human serum albumin. The clone  
5 chosen for further work was sequenced from both directions to verify the correct insertion into the vector and the presence of a stop codon between EAG and the intein-affinity tag.

#### HSA-column

50 mg human serum albumin (HSA, Sigma) were immobilized on a 5 ml HiTrap NHS-activated column (Pharmacia Biotech) according to the manufacturer's instructions.

10 Based on the absorbance at 280 nm, 65% of the HSA was estimated to be immobilized on the column.

#### Expression and purification – general

16 ml of an over night culture of EAG4B in *E. coli* ER2566 were inoculated into 2 liters of LB-medium supplemented with 50 µg ampicillin per ml. The culture was incubated at  
15 37°C until an OD<sub>600</sub> in the range of 0.5-0.6 was reached. Thereafter, IPTG was added to a final concentration of 0.5 mM and the culture was incubated at 25°C over night.

The culture was harvested and the pellet was washed once in 50 ml PBS-D before it was resuspended in 40 ml of 20 mM Na phosphate, pH 7.0 (binding buffer). The resuspension was divided into two tubes and 10 mg lysozyme were added to each tube. After 2 hrs. of  
20 incubation at 37°C, the tubes were frozen at -20°C over night.

The lysate was sonicated to fragmentize the DNA, centrifuged and the supernatant was filtered through a 0.45 µm filter.

The column was washed with 30 ml binding buffer before the sample (corresponding to 1 liter of the over night culture) was applied onto the column. Thereafter, the column was  
25 washed with 50 ml binding buffer and the A<sub>280</sub> value was confirmed to be below 0.010. Bound protein was eluted in 0.1 M glycine-HCl, pH 3.0. The first 4.5 ml were not collected while the next 10 ml were collected as 1 ml fractions, which were neutralized by addition of 100 µl 1 M Tris-buffer, pH 8.0. Then, the column was regenerated by addition of binding buffer and stored at +4°C in the presence of sodium azide.

30 The A<sub>280</sub> value was determined and the fractions containing protein, normally fractions 1 to 5 or 6 were pooled and dialyzed 3 times against 4 liters of PBS-D. Thereafter, the protein was concentrated by extracting water from the dialysis tubing with PEG 30 000.

From an over night culture of 2 liters, this procedure yielded 5-6 mg of an N-terminal fraction of the EAG protein, designated EAG4B and comprising the amino acid sequence recited above as SEQ. ID. NO: 1. In this sequence, the first amino acid presented in bold originates from the vector.

- 5 The nucleotide sequence of the fragment encoding EAG4B where the coding sequence starts from nucleotide 38(A) and ends at nucleotide 577(C) is shown below (SEQ. ID. NO: 7).

SEQ. ID. NO: 7:

	10	20	30	40	50
10					
	1 AAATAATTTTGTTTAACTTTAAGAAGGAGATATAACCATGGCTCTAGATG				
	51 CTACAACGGTGTTAGAGCCTACAACAGCCTTCATTAGAGAAGCTGTTAGG				
	101 GAAATCAATCAGCTGAGTGATGACTACGCTGACAATCAAGAGCTTCAGGC				
	151 TGTTCCTTGCTAATGCTGGAGTTGAGGCACTTGCTGCAGATACTGTTGATC				
15	201 AGGCTAAAGCAGCTCTTGACAAAGCAAAGGCAGCTGTTGCTGGTGTTTCAG				
	251 CTTGATGAAGCAAGACGTGAGGCTTACAGAACAAATCAATGCCTTAAGTGA				
	301 TCAGCACAAAAGCGATCAAAAGGTTGAGCTAGCTCTAGTTGCTGCAGCAG				
	351 CTAAGGTGGCAGATGCTGCTTCAGTTGATCAAGTGAATGCAGCCATTAAT				
	401 GATGCTCATAAGCTATTGCGGACATTACAGGAGCAGCCTTGTTGGAGGC				
20	451 TAAAGAAGCTGCTATCAATGAACTAAAGCAGTATGGCATTAGTGATTACT				
	501 ATGTGACCTTAATCAACAAAGCCAAACTGTTGAAGGTGTCAATGCGCTT				
	551 AAGGCAAAGATTTTATCAGCTCTACCGTAGCTCGAGCCCCGGTGCTTTGC				

### Example 2. Preparation of antigens derived from SFS

- 25 The SFS protein from *S. equi* subsp. *equi* has previously been described by Lindmark and Guss (1999) (Ref. 12) and in WO 00/37496. The GeneBank accession number for the nucleotide sequence of the *sfs* gene is AF 136451.

A C-terminal fragment of this protein was produced as follows:

- 30 The 3' end of the *sfs* gene was PCR amplified using the Taq DNA polymerase (Amersham) and chromosomal DNA from *S. equi* strain 1866 as template and the synthetic oligonucleotides
- OSFS25: 5'-GGTCCCATGGCAACTCCGAATTTAGAAGGA-3' (SEQ. ID. NO: 8) and
- OSFS23: 5'-CAGACTCGAGGTCGGGATTGTAAGAATAG-3' (SEQ. ID. NO: 9) as
- primers. The PCR procedure was performed in 100 µl under standard conditions as regards
- 35 buffer, template and primer concentration. The PCR cycles were performed under the following conditions: 1 min. of denaturation at 94°C, 30 sec. of annealing at 40°C, and 2 min. of extension at 72°C, which were repeated as 25 cycles. After amplification, the PCR product

was purified using phenol extractions, chloroform extractions and EtOH precipitation. The purified DNA was cleaved with the restriction enzymes *Nco*I and *Xho*I after which the DNA was purified as described above.

The DNA obtained above was ligated into the plasmid vector pTYB4 [(New England Biolabs, Beverly, MA, USA (NEB Inc.)), which previously had been digested with the same restriction enzymes and treated with alkaline phosphatase. After ligation (using the ReadyToGo ligation kit, Amersham), the DNA sample was electrotransformed into the *E. coli* strain ER2566 and spread on LAA-plates (Luria Bertani agar plates supplemented with ampicillin, final conc. 50 µg/ml). After incubation over night at 37°C, clones harboring plasmids with inserts were isolated and the presence of the correct insert was verified by DNA sequencing. One of the clones thereby obtained, called SFSC1, was chosen for production of the C-terminal part of SFS.

The vector used is a part of an *E. coli* expression and purification system called IMPACT<sup>T</sup> T7 (NEB Inc.). Briefly, following the manufacturer's instructions, the clone SFSC1 was grown at 37 °C in Luria Bertani growth medium supplemented with ampicillin (final conc. 50 µg/ml). At an optical density (OD<sub>600</sub>) of ~ 0.6, the growth medium was supplemented with IPTG (final conc. 0.4 mM) and the growth temperature was shifted to 20°C. After incubation over night, the cells were harvested and resuspended in a buffer [20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 0.1 mM EDTA, and 0.1 % Triton X100], lysed by freezing and thawing, and after centrifugation, the supernatant was sterile filtered and applied to a chitin column.

The column was extensively washed using the same buffer as above and was subsequently treated with cleavage buffer [20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.1 mM EDTA, and 30 mM dithiothreitol (DTT)]. The reducing conditions in the cleavage buffer induce an intein-mediated self-cleavage that releases the SFS part from the column while the intein-chitin-binding part remains bound. The eluted sample containing the product SFSC1 was dialysed against phosphate-buffered saline [PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.4 mM KH<sub>2</sub>PO<sub>4</sub> (pH 7.4)] and concentrated. The amount of protein obtained was determined and the quality checked using SDS-PAGE.

After the intein part has been cleaved off from SFSC1, the purified protein has an amino acid sequence as recited below as SEQ. ID. NO: 10 except that the amino acid residues in bold are residues that correspond to the amino acid residues encoded by the pTYP4 vector, while remaining amino acid residues originate from the SFS protein.

SEQ. ID. NO: 10:

MATPNLEGNN RKNPLKGLDG ENKPKEDLDG KGLSGENDES PKLKDEHPYN  
HGRRDGYRVG YEDGYGGKKH KGDYPKRFDE SSPKEYNDYS QGYNDNYGNG  
YLDGLADRGG KRGYGYSYNP DLEPG.

**Example 3. Preparation of antigens derived from FNZ**

5 Protein FNZ from *S. equi* subsp. *zooepidemicus* has previously been described by Lindmark et al (1996) (Ref. 9), Lindmark (1999) (Ref. 13), Lindmark et al (1999) (Ref. 11) and Lindmark et al (2001) (Ref. 14). The EMBL accession number for the nucleotide sequences of the *fnz* gene is X99995. A similar truncated protein called FNE is also expressed by strains of *S. equi* subsp. *equi* [Lindmark et al (2001), Ref. 14]. The GeneBank accession  
10 number for the nucleotide sequence of the *fne* gene is AF360373. The construction of a clone called pT2fnzN encoding the N-terminal part of FNZ has previously been described by Lindmark et al 2001 (Ref. 14). Briefly, the clone pT2fnzN was constructed as follows using PCR amplification with the forward primer  
OFNZ1: 5'-ACCATGGCTAGCGCAGAGCAGCTTTATTATGGGT-3' (SEQ. ID. NO: 11),  
15 and the reverse primer  
OFNZ2: 5'- ATACCCGGGATATCCTTCGGTACTACCATAGT-3' (SEQ. ID. NO: 12). Chromosomal DNA from subsp. *zooepidemicus* strain ZV was used as the template and the 5' end of the *fnz* gene was amplified. The PCR fragment obtained was cleaved with restriction endonucleases *NheI* and *SmaI*, followed by ligation into the corresponding restriction  
20 endonuclease sites in the expression vector pTYB2 (NEB). The ligated DNA was electrotransformed into *E. coli* strain ER2566. Plasmids harboring inserts were isolated from transformants and verified by DNA sequencing. One such clone is designated pT2fnzN. The production in *E. coli* and purification of the N-terminal part of FNZ, called FNZN, were performed as described above for protein SFSC1 and these steps are also described in  
25 Lindmark et al (2001) (Ref. 14).

The amino acid sequence of FNZN comprises then amino acid residues 32-337 of the amino acid sequence of the protein FNZ recited above as SEQ. ID. NO: 2. The corresponding amino acid sequence of FNZN is shown below as SEQ. ID. NO: 13.

30 SEQ. ID. NO: 13  
M A S A E Q L Y Y G W N D G T R Q S S P  
Y F L Y V S P K N A P K R E L K D E Y V  
V Y C F N K K L Y W P D Q W E S I Y S N  
F N D I R S P Y N D L P V Y E K K L G Y  
35 D G I F K Q Y A P D Y K K D I S D I A S

5       A L V A V L S N G Y P T N K S Q L S T S  
       Y H L N N D S S R K V T Q L A I W Y F S  
       D S L T K E Y L K D T G G Y N L N D M E  
       K K A L D F L I S K G E D S K L K S E Q  
       S N Y S L D I Y V Y Q S G G H D H M K D  
       Y Q N L L G S T L I P K E P L K P Q L G  
       G F S G H N G N G L S G L E G G S S G S  
       Q E T N E D G K K G L I G F H G G L S G  
       S E G K R D P L P G L K G E A G A P D T  
 10       P Q K P N D P L Q G L E G G N S P I V E  
       Q N Y G S T E G Y G

In the previously recited amino acid sequence (SEQ. ID. NO: 2) of protein FNZ, amino acids shown in bold originate from the protein FNZ in *S. equi* subspecies

15 *zooepidemicus*, while those shown in normal type are derived from the expression vector construct. In SEQ. ID. NO:13, the first three and the last amino acid residues originate from the vector.

#### Example 4. Preparation of antigens derived from the protein SEC

The genome of *S. equi* is accessible ([www.sanger.ac.uk](http://www.sanger.ac.uk)) for computer analysis. By  
 20 using earlier published sequences of virulence factors or potential virulence factors from pathogenic streptococci and staphylococci, it is possible to screen the genome of *S. equi* for the presence of similar genes. By using the soft ware program BLAST and searching for open reading frames encoding a protein similar to the collagen-binding protein CNA of *Staphylococcus aureus* (WO 92/07002), it was possible to identify a hypothetical gene of  
 25 1971 nucleotides in the *S. equi* genome encoding a protein of 657 amino acids. (Ref. 25, Lannergård et al. (2003)). This protein, hereafter termed protein SEC (SEQ. ID. NO: 4), encodes an N-terminal signal sequence (amino acid sequence 1-26) followed by a region, which displays collagen-binding. The C-terminal part of protein SEC contains all typical features of cell surface proteins from streptococci, like putative wall anchoring and membrane  
 30 spanning regions, as well as a motif corresponding to the LPDTG motif (SEQ. ID. NO: 14) of the protein SEC.

The nucleotide sequence of the *sec* gene (later renamed to *cne*) encoding the protein SEC (the accession no. of the *cne* is AY193773). is shown below as SEQ. ID. NO: 15.

SEQ. ID. NO: 15:

1

TTGAAACAACCTGACAAAGATCGTTAGTGTGGTCTTGTTGCTGGTCTTTACCCCTTAG  
TGCTAGCCTGCACAAGGTTTCGGGCAACTAATCTTAGTGACAACATCACATCATTG  
ACGGTTGCTTCTTCATCACTCCGAGATGGAGAGAGAACGACGGTAAAGGTTGCGT  
TTGATGACAAAAACAGAAAATCAAGGCAGGGGATACGATAGAGGTCACCTGGC  
CTACAAGTGGTAATGTCTACATTCAGGGCTTTAATAAAACCATAACCGCTTAATAT  
TAGAGGGGTAGATGTTGGTACCTTGGAGGTCACGCTAGACAAGGCTGTTTTACACA  
TTCAATCAAAATATTGAAACAATGCATGATGTCTCTGGTTGGGGAGAGTTTGATA  
TTACTGTTAGAAATGTGACACAAACCACCGCTGAAACATCAGGAACGACCACAG  
TAAAGGTAGGCAATCGCACTGCTACTATCACTGTTACTAAGCCTGAGGCAGGCAC  
TGGTACCAGCTCATTTTTATTATAAGACTGGTGATATGCAGCCCAATGATACTGAG  
CGTGTGAGATGGTTCCTGCTGATTAACAACAACAAGGAATGGGTGGCCAATACTG  
TTACAGTCGAAGACGATATTCAAGGTGGTCAAACCTTGGATATGAGCAGCTTTGA  
CATCACCGTATCTGGTTATCGTAACGAGCGCTTCGTTGGGAAAACGCTCTGACA  
GAGTTTCATACAACATTTCCAAATTCTGTCAATTACGGCAACAGATAATCACATTA  
GTGTGCGGTTAGATCAATATGATGCCTCACAAAACACTGTCAACATTGCTTATAA  
GACAAAGATAACGGACTTTGACCAAAAAGAATTTGCCAACAACAGTAAAATCTG  
GTACCAGATTTTATACAAGGATCAGGTATCGGGTCAAGAGTCAAACCACCAAGTA  
GCCAATATCAATGCTAACGGCGGGGTTGATGGCAGTCGCTATACCAGCTTTACTG  
TCAAGAAAATTTGGAATGACAAGGAAAATCAAGACGGTAAGCGTCCAAAGACTA  
TTACTGTTCACTTTACGCCAATGATCAGAAAGTTAATGATAAGACCATTGAATT  
GAGTGATACTAATAGCTGGCAAGCAAGTTTTGGTAAGCTGGATAAGTATGACAGT  
CAGAACCAAAAAATTACCTACAGTGTCAAGGAAGTGATGGTTCCTGTTGGCTACC  
AATCGCAGGTTGAGGGGGATAGTGGAGTAGGATTTACCATTACCAACACCTATAC  
ACCAGAGGTCATTAGCATTACCGGTCAAAAAACTTGGGACGACAGGGAAAACCA  
AGACGGTAAACGTCCTAAGGAGATTACGGTTCGTTTATTGGCAAATGACGCTGCA  
ACTGACAAGGTAGCAACTGCTTCAGAGCAAACCGGCTGGAAGTATACATTTACCA  
ATCTACCGAAATACAAAGATGGTAAACAGATCACCTACACGATCCAAGAGGACC  
CTGTGGCAGATTACACCACAACCATTACAGGGATTTGATATTACCAATCATCATGA  
GGTAGCCTTGACCAGCCTAAAGGTCATCAAGGTTTGGAAATGATAAGGACGATTAT  
TACCATAAACGTCCCAAGGAGATTACCATTTTGCTAAAGGCAGATGGCAAGGTGA  
TTCGTGAACATCAGATGACACCGGATCAGCAAGGAAAATGGGAATACACCTTTG  
ACCAGCTGCCGGTCTATCAGACAGGCAAGAAAATCAGCTACAGCATTGAGGAAA  
AACAGGTTGCTGGCTATCAAGCCCCTGTCTATGAGGTTGATGAAGGCTTGAAGCA  
GGTCACTGTAACCAACACCCTTAACCCAAGCTACAAGCTGCCTGACACCGGAGGA  
CAAGGAGTGAAATGGTACCTGTTAATCGGTGGCGGTTTTATCATCGTCGCAATCC  
TTGTACTGATCAGCCTTTATCAAAAACACAAGCGCCATAACATGTCAAAACCA

1971

5

### Construction of recombinant protein SEC expressing collagen-binding activity

To express and purify the collagen-binding activity of protein SEC, two different constructions were made. The first construct, encoding the major part of protein SEC was made as follows. The primers

10

OSEC1:5: 5'-CATGCCATGGCAACTAATCTTAGTGACAACAT-3' (SEQ. ID. NO: 16)

and

OSEC3:3: 5'-CCGCTCGAGCTTGTAGCTTGGGTAAAGGGTGT-3' (SEQ. ID. NO: 17)

were used to PCR-amplify a DNA-fragment corresponding to a sequence from amino acid no.



27 to amino acid no. 615 in protein SEC (SEQ. ID. NO: 4) using *S. equi* subspecies *equi* 1866 chromosomal DNA as a template.

The second construct encoding the N-terminal part of protein SEC was made as follows. The primers OSEC1:5 and

- 5 OSEC2:3: 5'-CCGCTCGAGAAAGCTGGTATAGCGACTGCCAT-3' (SEQ. ID. NO: 18) were used to PCR-amplify a DNA-fragment corresponding to a sequence from amino acid no. 27 to amino acid no. 328 in protein SEC (SEQ. ID. NO: 4) using *S. equi* subspecies *equi* 1866 chromosomal DNA as a template.

- Both PCR amplifications were performed using ReadyToGo™ PCR beads (Amersham Pharmacia Biotech Inc) and the PCR apparatus MiniCycler™ (MJ Research, Inc, MA, USA) using a program comprising: step 1: 95°C, 1 min; step 2: 95°C, 30 sec.; step 3: 46°C, 15 sec.; and step 4: 72°C, 2 min; repeated as 25 cycles from step 2 to step 4. After PCR amplification, the respective PCR-products were purified and digested with restriction endonucleases *NcoI* and *XhoI* and ligated into the pTYB4-vector (NEB) digested previously with the same enzymes. One µl of the respective ligation mixture was transformed into *E. coli* ER2566. After IPTG induction, *E. coli* clones expressing collagen-binding were identified by colony screening using <sup>125</sup>I-labeled collagen (collagen S, type I obtained from Boehringer Mannheim). Several clones of both types of constructions expressing collagen-binding were identified and further characterized. One of these clones called pSEC 2.16 harboring the PCR-fragment and originating from the PCR using OSEC 1:5 and 3:3 was chosen after DNA sequencing of the insert for production of a recombinant collagen-binding protein called protein SEC 2.16 and comprising amino acid residues 27 - 616 of SEQ. ID. NO: 4. The nucleotide sequence of this insert encoding the recombinant collagen-binding protein SEC2.16 is shown below as SEQ. ID. NO: 19. The nucleotides shown in bold represent nucleotides originating from the vector.

SEQ. ID. NO: 19: Insert of pSEC2.16

ATGGCAACTAATCTTAGTGACAACATCACATCATTGACGGTTGCTTCTTCATCACTCCGAGATGGA  
GAGAGAACGACGGTAAAGGTTGCGTTTGATGACAAAAACAGAAAATCAAGGCAGGGGATACGA  
TAGAGGTCACCTGGCCTACAAGTGGTAATGTCTACATTCAGGGCTTTAATAAAACCATAACCGCTTA  
ATATTAGAGGGGTAGATGTTGGTACCTTGGAGGTCACGCTAGACAAGGCTGTTTTACATTCAAT  
CAAAATATTGAAACAATGCATGATGTCTCTGGTTGGGGAGAGTTTGATATTACTGTTAGAAATGTG  
ACACAAACCACCGCTGAAACATCAGGAACGACCACAGTAAAGGTAGGCAATCGCACTGCTACTAT  
CACTGTTACTAAGCCTGAGGCAGGCACTGGTACCAGCTCATTATTATAAGACTGGTGATATTCA  
GCCAATGATACTGAGCGTGTGAGATGGTTCCTGCTGATTAACAACAACAAGGAATGGGTGGCC  
AATACTGTTACAGTCGAAGACGATATTCAAGGTGGTCAAACCTTGGATATGAGCAGCTTTGACATC  
ACCGTATCTGGTTATCGTAACGAGCGCTTCGTTGGGGAAAACGCTCTGACAGAGTTTCATACAAC  
ATTTCCAAATTCTGTCAATTACGGCAACAGATAATCACATTAGTGTGCGGTTAGATCAATATGATGC  
CTCACAAAACACTGTCAACATTGCTTATAAGACAAAGATAACGGACTTTGACCAAAAAGAATTTGC

CAACAACAGTAAAATCTGGTACCAGATTTTATACAAGGATCAGGTATCGGGTCAAGAGTCAAACC  
 ACCAAGTAGCCAATATCAATGCTAACGGCGGGGTTGATGGCAGTCGCTATACCAGCTTTACTGTC  
 AAGAAAATTTGGAATGACAAGGAAAATCAAGACGGTAAGCGTCCAAAGACTATTACTGTTTCAGCTT  
 TACGCCAATGATCAGAAAGTTAATGATAAGACCATTGAATTGAGTGATACTAATAGCTGGCAAGCA  
 AGTTTTGGTAAGCTGGATAAGTATGACAGTCAGAACCAAAAAATTACCTACAGTGTCAAGGAAGT  
 GATGGTTCCTGTTGGCTACCAATCGCAGGTTGAGGGGGATAGTGGAGTAGGATTTACCATTACCA  
 ACACCTATACACCAGAGGTCATTAGCATTACCGGTCAAAAAACTTGGGACGACAGGGAAAACCAA  
 GACGGTAAACGTCCTAAGGAGATTACGGTTCGTTTATTGGCAAATGACGCTGCAACTGACAAGGT  
 AGCAACTGCTTCAGAGCAAACCGGCTGGAAGTATACATTTACCAATCTACCGAAATACAAAGATG  
 GTAAACAGATCACCTACACGATCCAAGAGGACCCTGTGGCAGATTACACCACAACCATTTCAGGGA  
 TTTGATATTACCAATCATCATGAGGTAGCCTTGACCAGCCTAAAGGTCATCAAGGTTTGGGAATGAT  
 AAGGACGATTATTACCATAAACGTCCCAAGGAGATTACCATTTTGCTAAAGGCAGATGGCAAGGTG  
 ATTCGTGAACATCAGATGACACCGGATCAGCAAGGAAAATGGGAATACACCTTTGACCAGCTGC  
 CGGTCTATCAGGCAGGCAAGAAAATCAGCTACAGCATTGAGGAAAAACAGGTTGCTGGCTATCAA  
 GCCCTGTCTATGAGGTTGATGAAGGCTTGAAGCAGGTCACTGTAACCAACACCCTTAACCCAAG  
 CTACAAGCTCGAGCCCGGG

The amino acid sequence of the recombinant collagen-binding protein SEC2.16 encoded by the insert of pSEC2.16 is shown below as SEQ. ID. NO: 20. The amino acids shown in bold represent amino acids originating from the vector.

5

SEQ. ID. NO: 20: Protein SEC2.16

MATNLSDNITSLTVASSSLRDGERTTVKVAFDKKQKIKAGDTIEVTWPTSGNVYIQGFNKTIPLNIRGV  
 DVGTLLEVTLDAVFTFNQNIETMHDVSGWGEFDITVRNVQTTAETSGTTTTVKVGNRTATITVTKPEA  
 GTGTSSFYYKTGDIQPNTERVRWFLNNNKEWVANVTVEDDIQGGQTLDMSSFDTITVSGYRNER  
 FVGENALTEFHTTFPNSVITATDNHISVRLDQYDASQNTVNIAYKTKITDFDQKEFANNSKIWYQILYKD  
 QVSGQESNHQVANINANGGVDGSRYTSFTVKKIWNKDKENQDGKRPKTITVQLYANDQKVNDKTIELS  
 DTNSWQASFGKLDKYDSQNQKITYSVKEVMVPVGYQSQVEGDSGVGFTITNTYTPEVISITGQKTWD  
 DRENQDGKRPKETVRLLANDAATDKVATASEQTGWKYTFTNLPKYKDQKITYTIQEDPVADYTTTIQ  
 GFDITNHHEVALTSLKVIKVVNDKDDYYHKRPKEITILLKADGKVIREHQMTDPQQGKWEYTFDQLP  
 VYQAGKKISYSIEEKQVAGYQAPVYEVDGLKQVTVTNTLNPSYKLEPG

- 10 The other clone obtained above that was chosen for further studies was called pSEC 1.18 and harbored the PCR-fragment originating from the PCR using OSEC 1:5 (SEQ. ID. NO. 16) and 2:3 (SEQ. ID. NO. 18). After DNA sequencing of the insert of pSEC 1.18, this clone was used for production of a recombinant collagen-binding protein called protein SEC 1.18. This protein SEC 1.18 comprises the amino acid sequence shown below as SEQ. ID. NO. 22. The
- 15 corresponding nucleotide sequence is shown below as SEQ. ID. NO. 21. In this nucleotide sequence, the nucleotides shown in bold represent nucleotides originating from the vector.

SEQ. ID. NO. 21: Insert of pSEC1.18

ATGGCAACTAATCTTAGTGACAACATCACATCATTGACGGTTGCTTCTTCATCACTCCGAGATGGA  
 GAGAGAACGACGGTAAAGGTTGCGTTTGATGACAAAAACAGAAAATCAAGGCAGGGGATACGA  
 TAGAGGTCACCTGGCCTACAAGTGGTAATGTCTACATTCAGGGCTTTAATAAAACCATAACCGCTTA  
 ATATTAGAGGGGTAGATGTTGGTACCTTGGAGGTCACGCTAGACAAGGCTGTTTTACATTCAAT  
 CAAAATATTGAAACAATGCATGATGTCTCTGGTTGGGGAGAGTTTGATATTACTGTTAGAAATGTG

ACACAAACCACCGCTGAAACATCAGGAACGACCACAGTAAAGGTAGGCAATCGCACTGCTACTAT  
 CACTGTTACTAAGCCTGAGGCAGGCACTGGTACCAGCTCATTTTATTATAAGACTGGTGATATGC  
 AGCCCAATGATACTGAGCGTGTGAGATGGTTCCTGCTGATTAACAACAACAAGGAATGGGTGGC  
 CAATACTGTTACAGTCGAAGACGATATTCAAGGTGGTCAAACCTTGGATATGAGCAGCTTTGACAT  
 CACCGTATCTGGTTATCGTAACGAGCGCTTCGTTGGGGAAAACGCTCTGACAGAGTTTCATACAA  
 CATTTCCAAATTCTGTCAATTACGGCAACAGATAATCACATTAGTGTGCGGTTAGATCAATATGATG  
 CCTCACAAAACACTGTCAACATTGCTTATAAGACAAAGATAACGGACTTTGACCAAAAAGAATTTG  
 CCAACAACAGTAAATCTGGTACCAGATTTTATACAAGGATCAGGTATCGGGTCAAGAGTCAAAC  
 CACCAAGTAGCCAATATCAATGCTAACGGCGGGGTTGATGGCAGTCGCTATACCAGCTTTCTCGA  
 GCCCCGGG

The amino acid sequence of the recombinant collagen-binding protein SEC1.18 encoded by the insert of pSEC1.18 is shown below as SEQ. ID. NO: 22. The amino acids shown in bold represent amino acids originating from the vector.

5

SEQ. ID. NO: 22: Protein SEC1.18.

MATNLSDNITSLTVASSSLRDGERTTVKVAFDKKQKIKAGDTIEVTWPTSGNVYIQGFNKTIPLNIRGV  
 DVGTLLEVTLDKAVFTFNQNIETMHDVSGWGEFDITVRNVQTTAETSGTTTVKVGNRATITVTKPEA  
 GTGTSSFYYKTGDMQPNTERVRWFLINNKEWVANTVTVEDDIQGGQTLDMSSFDTVSGYRNER  
 FVGENALTEFHTTFPNSVITADNHISVRLDQYDASQNTVNIA YKTKITDFDQKEFANNSKIWYQILYKD  
 QVSGQESNHQVANINANGGVDGSRYTSFLEPG

#### **Production of protein SEC 1.18 and protein SEC 2.16**

10

Since the vector used to construct the pSEC1.18 and pSEC 2.16 is a part of the IMPACT-system (NEB), the expression and purification of protein SEC1.18 and protein SEC2.16 were performed as described for protein SFSC1 and protein FNZN. After purification, protein SEC 1.18 and protein SEC 2.16 were analysed by SDS-PAGE using the Phast-System (Amersham Pharmacia) and 8-25% gradient gels under reducing conditions (SDS buffer strips, boiling the samples in a sample buffer containing SDS and  $\beta$ -mercapto-ethanol before applying the samples on the gels). The results show that the purified protein SEC 1.18 and protein SEC 2.16, approx. had relative molecular weights corresponding to their calculated molecular weights.

15

#### **Collagen-binding activity.**

20

The purified proteins SEC 1.18 and SEC 1.16 were also analysed for their ability to bind to collagen. First, the proteins were run on an SDS-PAGE 8-25% gel (Amersham Pharmacia) under reducing conditions. After the electrophoresis was completed, the proteins were transferred (by diffusion) to a nitro-cellulose membrane. The membrane was blocked in a solution (PBS-T) containing PBS supplemented with TWEEN 20 (final concentration 0.5%) and casein 0.5% (final conc.) for 1 hr. at room temperature (RT). After washing with PBS-T (casein omitted), the membrane was transferred to a solution of PBS-T containing  $^{125}$ I-labeled

25

collagen. After 4 hrs. of incubation at RT under gentle agitation, the membrane was extensively washed using PBS-T (casein omitted) and subjected to autoradiography using Biomax MS (Kodak) film. The results showed that in contrast to unrelated proteins used as control (size marker proteins), both protein SEC 1.18 and protein SEC 2.16 bound collagen.

5           **Occurrence of the *sec* gene in *S. equi***

Using chromosomal DNA from various strains of *S. equi* subspecies *equi* and subspecies *zooepidemicus* as template and the primer combinations OSEC1:5 (SEQ. ID. NO: 16) and OSEC 2:3 (SEQ. ID. NO: 18) or OSEC1:5 (SEQ. ID. NO: 16) and OSEC 3:3 (SEQ. ID. NO: 17), the presence of the *sec* gene was investigated. The PCR conditions applied were  
10 the same as described above in the section "Construction of recombinant protein SEC expressing collagen-binding activity". The result showed that the *sec* gene was present in all tested strains in both subspecies. This is an important finding, since a vaccine containing an antigen, which is present in both subspecies, gives the possibility to broaden the application of the invention resulting in a vaccine active against infections of both subspecies.

15

**Further search for vaccine components.**

The genome of *S. equi* is accessible ([www.sanger.ac.uk](http://www.sanger.ac.uk)) for computer analysis. As for protein SEC, it is possible to further screen the genome of *S. equi* for genes encoding earlier published sequences of virulence factors or potential virulence factors from pathogenic  
20 streptococci and staphylococci. Use of the soft ware program BLAST resulted in the identification in *S. equi* of additional genes encoding potential virulence factors similar to *Streptococcus pyogenes*: C5a peptidase (gi:14195215, sp, P58099, SCA2 STRPY), SclB (emb: CAC33776.1), collagen-like protein similar to (ref: NP358996.1), speK (ref: NP 438166.1), exotoxin I (gi:16923748, gb:AAL31571.1, AF438524.1), streptodornase (emb:CAA59264.1),  
25 a DNA entry nuclease (ref: NP 346391.1), trypsin resistant surface T6 protein (sp:P18481, TEE6 STRPY), M and M-like proteins, other fibronectin-binding proteins and other.

Furthermore, the computer analyses revealed several hypothetical proteins displaying a structure which is found in cell wall-associated proteins in Gram-positive cocci (Schneewind et al 1995, Ref. 15). At the N-terminal end of these proteins, a signal sequence is found and at the C-terminal end, a motif LPXTG corresponding to the LPDTG motif (SEQ. ID. NO: 14) of the SEC protein is present, which is followed by a stretch of hydrophobic amino acids ending with a few charged amino acids constituting the membrane-spanning region. These proteins should also be considered as potential targets to be included in an efficient vaccine against *S. equi* infections.

Based on considerations as discussed above, two proteins have been prepared and evaluated as disclosed in the following Examples 5 and 6.

#### Example 5. Preparation of protein SclC and protein SCLC1

The amino acid sequence of *Streptococcus pyogenes* protein SclB was used to screen the genome of *S. equi* ([www.sanger.ac.uk](http://www.sanger.ac.uk)) using the software program BLAST. As a result of this screening, an open reading frame was identified, which encodes a protein called SclC, which is similar to SclB. The protein SclC comprises an amino acid sequence as shown as SEQ. ID. NO: 23 below.

SEQ. ID. NO: 23:

1  
 20 MTNKT KRTGLVRKYGACSA AIALAALASLGAGKAVKAD  
 QPAALKYPEPRDYFLHTREGDVIYDEEDIKRYFEDLEAY  
 L TARLG GIDKKVEEAAQKPGIPGPTGPQGPKGDKGDPG  
 APGERGPAGPKGDTGEAGPRGEQGPAGQAGERGPKG  
 PGAPGPKGEKGD TGA VGPKEKGD TGA TGPKGDKGER  
 25 GEKGEQGRGEKGEQGRGEKGEQKPKGDQ GKDTKP  
 SAPKAPEKAPAPKAPKASEQSSNP KAPAPKSA PSKSA  
 PTGQKAALPATGEINHPFFTLAALSVIASVGVLT LK GK  
 KD  
 302

30 The nucleotide sequence of the gene sclC encoding protein SclC is shown below as SEQ. ID. NO: 24.

SEQ. ID. NO: 24:

35 1  
 atgacaaacaaaacaaagcgtacaggattggtacgcaagtagcgtgctcagcagctatgccttagcagctcttgaagcctgg  
 gaggcaggtaaagcagtaaaggcagaccagccagcagcactaaaatatccagaacctagagactattttctcactactcgtgaaggat  
 gttatttatgatgaggatataaaaagatattttgaggattagaagcctatttaacagctagacttggtgggattgataaaaagtagaagaa  
 40 gctgccccaaaagccaggtattccaggtcctactggccctcaaggtcctaagggagacaaaaggagatccaggtgcccctggtgagcgc  
 ggtccagctggaccaaagggcgatagggcggaagccggaccaagaggtgagcaaggccagccggacaagctggagaacgtgg  
 accaaaaggagatccaggtgctccaggtcctaaggtgaaaaggggtgatactggtgcagttggtcctaaggtgaaaaggtgatacc

ggagcaaccggaccaaagggagacaagggcgaaacgcggtgaaaaaggcgagcaaggccaacgtggcgaaaaaggcgagcaa  
 ggccaacgcggtgaaaaaggcgagcaaaaaccaaaagggtgatcaaggaaaagatacaaaaccatcagctccaaaagcacctgaaa  
 aggctcctgcacaaaagctcctcaaaaggcttcagagcagtcacatctaataagcaccagctcctaagtcagcaccaagcaaatcagc  
 5 ggccaacaggtcaaaaagcagccctaccagcaacaggggaaatcaaccacccattcttcacccttcagctcttagtgcacgcta  
 gcgtaggcgtcctaactctaaaaggaaaaaaagactaa

909

To express and purify the major part of the SclC protein, the following construction was made. The primers

OSCL2:5: 5'- CATGCCATGGACCAGCCAGCAGCACTAAAATAT-3' (SEQ. ID. NO: 25)

10 and OSCL3:3: 5'- CCGCTCGAGGGCTGCTTTTTGACCTGTTGGT-3' (SEQ. ID. NO: 26)

were used to PCR-amplify a DNA-fragment corresponding to amino acid 38 to amino acid 269 in protein SclC using *S. equi* subspecies *equi* 1866 chromosomal DNA as a template.

PCR amplification was performed using ReadyToGo™ PCR beads (Amersham Pharmacia Biotech Inc) and the PCR apparatus MiniCycler™ (MJ Research, Inc, MA, USA) using a

15 program of step 1: 95°C, 1 min; step 2: 95°C 30 sec.; step 3: 50°C 15 sec.; step 4: 72°C, 1 min; repeated as 24 cycles from step 2 to step 4. After PCR amplification, the respective

PCR-products were purified and digested with restriction endonucleases *NcoI* and *XhoI* and ligated into the pTYB4-vector (NEB) previously digested with the same enzymes. One µl of

the respective ligation mixture was transformed into *E. coli* ER2566. *E. coli* clones harboring

20 the *sclC* fragment were identified by DNA sequencing of the inserted fragments.

Of these clones, one clone called pSclC1 and harboring the PCR-fragment originating from the PCR using OSCL2:5 (SEQ. ID. NO: 25) and OSCL3:3 (SEQ. ID. NO: 26), was chosen for production of a recombinant SclC protein called protein SCLC1. This protein SCLC1 comprises the amino acid sequence shown below as SEQ. ID. NO: 27.

25

SEQ. ID. NO: 27:

MDQPAALKYPEPRDYFLHTREGDVIYDEEDIKRYFEDLE  
 AYLTLARLGIDKKVEEAQKPGIPGPTGPQGPKGDKGD  
 30 PGAPGERGPAGPKGDTGEAGPRGEQGPAGQAGERGPK  
 GDPGAPGPKGEGKDTGAVGPKGEGKDTGATGPKGDKG  
 ERGEKGEQGRGEKGEQGRGEKGEQKPKGDQGKDT  
 KPSAPKAPEKAPAPKAPKASEQSSNPKAPAPKSAPSKS  
 AAPTGQKAALPEG  
 35

In the peptide sequence of the insert of pSclC1 encoding the recombinant protein SCLC1 (SEQ. ID. NO: 27), the amino acids shown in bold represent amino acids originating from the vector.

#### Production of protein SCLC1

Since the vector used to construct the pSCLC1 is a part of the IMPACT-system (NEB) the expression and purification of protein SCLC1 was performed as described for the other IMPACT-constructions (proteins SFSC1, FNZN, SEC 1.18 and SEC 2.16). After purification protein SCLC1 was analysed by SDS-PAGE using the Phast-System (Amersham Pharmacia) and 8-25% gradient gels under reducing conditions (SDS buffer strips, boiling the samples in a sample buffer containing SDS and beta-mercaptoethanol before applying the samples on the gels). The results showed that the purified protein had a relative molecular weight corresponding to its calculated molecular weight. The purified protein SCLC1 was then used to immunize mice and rabbits and was also used to screen convalescence sera from horses for measurements of antibody titers against SCLC1. This is illustrated in Example 6 below.

**Example 6. Immunization of rabbits using protein SCLC1 and Western blot**

Protein SCLC1 was used to immunize a rabbit. This work was done at the company Agrisera (Vännäs, Sweden). Using an ELISA format, where the SCLC1 protein was immobilized in microtiter wells, the immune serum obtained was analysed for presence of antibodies against SCLC1. The results showed that the immune sera could be diluted > 30 000 and still react with the SCLC1 protein (as compared to the pre-immune serum, which did not contain any significant level of antibodies against SCLC1).

In a Western blot analysis, the SCLC1 protein was also run on an SDS-PAGE gel using the Phast-System (Amersham Pharmacia) and 8-25% gradient gel under reducing conditions. After the electrophoresis had been completed, the SCLC1 protein was diffusion blotted to a nitrocellulose-membrane. After blocking, the membrane was divided and immune serum (dilution 1:10 000 in PBS-0.05%Tween 20) and pre-immune serum, respectively, were added and incubated for two hours at room temp. After washing in PBS-0.05%Tween 20, secondary antibodies (anti rabbit IgG, horseradish-labelled and developed in goat, Sigma) were used to detect rabbit antibodies directed against SCLC1. The result showed that the immune serum detected the immobilized SCLC1 protein efficiently, while no detection was seen using the pre-immune serum.

**Occurrence of sclC gene in strains of subsp. *equi* and subsp. *zooepidemicus***

Chromosomal DNA from different isolates of both subsp. was used as template for a PCR-analysis to study the occurrence of the sclC gene. The PCR primers used were OSCL2:5 (SEQ. ID. NO: 25) and OSCL3:3 (SEQ. ID. NO: 26). The result showed that a similar gene was present in all studied strains of subsp. *equi* and in a majority of strains in subsp. *zooepidemicus*.

In the following Examples 7-10, immunization and challenge of mice were performed as described below.

**Bacterial strain and growth:** *Streptococcus equi* subsp. *equi* was isolated from a horse with strangles. After one passage in a mouse (inoculated intranasally and reisolated from a submandibular gland), the bacteria were stored at  $-80^{\circ}\text{C}$ . To prepare an inoculum, stored bacteria were first grown on a blood agar plate, subcultured overnight at  $37^{\circ}\text{C}$  in Todd Hewitt medium containing 1% (w/v) yeast extract. Six ml of this culture were taken into 50 ml of Todd Hewitt /Yeast extract medium (BBL, Becton Dickinson) containing 10% (v/v) equine, i.e. horse, serum (heat inactivated) and incubated at  $37^{\circ}\text{C}$  for 4 hrs. Bacteria grown this way were used in animal challenge experiments.

**Mice and experimental design:** NMRI outbred female mice (Møllegaard, Denmark) having a body weight of about 20 g, were individually marked, and kept in groups of four in each cage. Daily, they were weighed and nose impressions onto a BG plate (sheep blood and gentian violet) were taken to quantify bacterial growth. After incubation at  $37^{\circ}\text{C}$  in 5 %  $\text{CO}_2$  for 24 hrs, the amount of haemolytic colonies was determined according to a scale of 0-3. A score of 3 means confluent growth of *Streptococcus* subsp *equi* on the whole plate and a score of 0 indicates 0-4 colonies.

**Bronchoalveolar lavage (BAL) and nasal wash (NW) for determination of mucosal IgA responses:** BAL was performed by infusing and withdrawing a solution consisting of 1 ml ice-cold PBS containing protease inhibitor (Complete, Roche) three times into the trachea. NW was performed by rinsing the nasal cavity with 0.5 ml ice-cold PBS containing protease inhibitor. The BAL and NW samples were kept on ice before storage at  $-80^{\circ}\text{C}$ .

**Statistical analysis:** Differences between the groups were evaluated by using the Student *t*-test, where  $p < 0.05$  was considered to be statistically significant.

**Example 7: Immune response in mice subjected to infection with *Streptococcus* subsp. *equi***

Mice ( $n=19$ ) were infected with *Streptococcus* subsp. *equi*. Ten  $\mu\text{l}$  of bacteria containing  $1 \times 10^6$  CFU were given intranasally to anaesthetized mice, viz. 5  $\mu\text{l}$  in each nostril. After two weeks, the mice were sacrificed and blood samples were taken. All mice had developed IgG antibodies against FNZN, SFSC1, and EAG4B. The results from eight mice are shown in Figures 1, 2, and 3 for FNZN, SFSC1, and EAG4B, respectively. These ELISA tests were performed according to a standard procedure. Briefly, wells on microtiter plates (Costar) were coated with the respective proteins at a concentration of 10  $\mu\text{g/ml}$ . Serum



dilutions from the mice samples were applied. After washing, rabbit antibodies against mice IgG conjugated with horse radish peroxidase (Dako) were added. To develop the color reaction, OPD tablets (Dako) were added according to instructions from the manufacturer.

**Example 8: Immune response in mice immunized with FNZN, SFSC1, and EAG4B**

Mice (n=24) were immunized subcutaneously on days 0, 7, 14, and 21. Twelve mice were given antigens (FNZN, SFSC1, and EAG4B) and adjuvant and twelve mice were given only adjuvant. The adjuvant used was EtxB provided by Dr Tim R. Hirst, University of Bristol. Each animal was given 12 µg of each antigen and 36 µg of EtxB at each occasion. On day 28, the mice were sacrificed and blood samples were taken. ELISA tests were performed as described above. All mice developed IgG antibodies against FNZN, SFSC1, and EAG4B. Samples from seven mice and a negative control are shown in Figures 4, 5, and 6 for FNZN, SFSC1, and EAG4B, respectively.

**Example 9: Immune response in mice vaccinated with SEC2.16**

Mice (n=5) were subcutaneously immunized on days 0, 7, 14, and 21. Each animal was given 12 µg of SEC2.16 in Freund's adjuvant. Serum samples were taken on day 28 and ELISA tests were performed as described above. In Figure 7, the production of IgG antibodies against SEC2.16 is shown.

**Example 10: Subcutaneous immunization with FNZN, SFSC1, and EAG4B followed by challenge with *Streptococcus equi* subsp *equi***

Mice (n=24) were subcutaneously immunized on days 0, 7, 14, and 21. Twelve mice were given antigens (FNZN, SFSC1, and EAG4B) and the adjuvant EtxB and twelve mice were given only adjuvant. Each animal was given 12 µg of each antigen and 36 µg of EtxB at each occasion. On day 28, the mice were infected with *Streptococcus equi* subsp. *equi*. Ten µl of bacteria containing  $1 \times 10^6$  CFU were given intranasally to anaesthetized mice. Animals that lost more than 15% of weight were killed. Mice that had survived or had lost less than 15% of weight are shown in Figure 8. It is evident from Figure 8 that non-vaccinated animals had a lower survival rate than vaccinated animals. On day 3, the p-value was  $< 0.05$ . From Figure 9, it is evident that in comparison to non-vaccinated animals, fewer bacteria grew in the noses of vaccinated animals; p-values obtained on days 2, 3, and 7 are 0.03, 0.02, and 0.01, respectively.

**Example 11. Intranasal immunization with FNZN, SFSC1, EAG4B and SEC2.16 followed by challenge with *Streptococcus equi* subsp. *equi***

One group of mice (n=24) was intranasally immunized on days 0, 7, 14, and 21 by administration of the antigens in 40 µl as several small doses in both nostrils. Twelve mice received the antigens FNZN, SFSC1, and EAG4B together with the adjuvant EtxB and twelve mice received only the adjuvant. Each animal was given 12 µg of each antigen together with 36 µg of EtxB at each occasion. On day 28, the mice were anaesthetized and infected with *Streptococcus equi* subsp. *equi* by intranasal administration of 10 µl of bacteria containing  $1 \times 10^6$  CFU (colony forming units).

Weight loss versus time is shown in Figure 10; on days 2, 3, 4, 5, and 8, the p-values were 0.04, 0.017, 0.005, 0.003, and 0.002, respectively. Nasal growth versus time is shown in Figure 11; on days 4 to 8, the p-value was <0.001. IgA antibody titers in BAL/NW and sera were determined by ELISA. Briefly, microtiter plates (Costar) were coated with 10 µg/ml of FNZN, SFSC1, and EAG4B. Serial dilutions of BAL/NWs were added to the wells. After washing, goat anti mouse IgA conjugated with horse radish peroxidase (Dako) was added. To develop the color reaction, OPD tablets (Dako) were added. From Figure 12 it is evident that BAL/NW samples from the animals contained IgA. Moreover, IgA against SFSC1 and EAG4B was also found in serum samples from some of the animals (data not shown).

Another group of mice (n=15) was immunized intranasally with SEC2.16 using Matrix (Ref. 20) as adjuvant and a control group was given the Matrix adjuvant alone (n=15). The animals were subjected to challenge infection with subsp. *equi*. The number of animals that died or lost more than 15% weight were different in the two groups; five vaccinated mice and 8 non-vaccinated died (Figure 18). This is not a statistically significant difference but is a tendency implying an effect of SEC2.16. It is likely that it would give a synergistic effect together with any or all of the other antigens mentioned above. Growth of subsp. *equi* in the noses was also studied. More bacteria were isolated from non-vaccinated mice than from vaccinated mice. On day 10, the score was 2.1 and 1.8 (on a scale from 0-3) in these groups, again implying an effect by vaccination with SEC2.16.

**Example 12. Immunization of horses**

Horses with no previous history of strangles and with low antibody titers against FNZ, SFS and EAG were selected. Four groups (A-D) of horses with three horses in each group were immunized as follows: A) EAG4B, FNZN and SFSC1 with EtxB as adjuvant, and given both intranasally and subcutaneously; B) As in group A but given only intranasally; C) as in A but without EtxB as adjuvant; and D) Only EtxB, and no antigens, given both

intranasally and subcutaneously. Each dose consisted of 35 µg of each antigen and 100 µg of EtxB in a volume of 2 ml for intranasal and/or subcutaneous immunization. Immunizations were given on days 0, 14, 28 and 42.

Serum samples were taken and analysed as follows: Microtiter wells (Costar) were coated overnight with 100 µl FNZN, SFSC1, or EAG4B, at 10 µg per ml overnight in phosphate buffered saline (PBS). The plates were then blocked with 2% (w/v) bovine serum albumin (BSA) for 1 hour at 37°C. After washing, horse sera were added to wells at a 20-fold dilution, followed by 2-fold serial dilutions. The plates were washed after 1 hour of incubation at 37°C. After washing, detection of antibody binding was performed with antibodies, diluted 1000x, against horse IgG, raised in rabbit and conjugated with HRP (Sigma Chemical Co). Development of a colorimetric reaction was achieved with OPD tablets (Dako, Denmark). Absorbance was determined spectrophotometrically at 492 nm.

In this way, IgG antibodies in sera of the immunized horses were determined. The results are shown in Figure 14. The log dilution of sera required to give an absorbance at a cut off value of 1.0 was calculated for each individual serum sample. Mean values (n=3) with standard errors are shown. Samples taken before (day 0) and after (day 56) immunizations are shown. The horses were divided into four groups A) (white bars): antigens + EtxB given both s.c. and i.n.; B) (striped bars): as group A but immunization only i.n.; C) (gray bars): As in group A but omitting EtxB; D) (black bars): control group given only EtxB both i.n. and s.c.

### **Example 13. Determination in ELISA of antibody titers from horses with or without a history of previous strangles**

Serum samples were taken from horses (n=16) without any previous or present signs of strangles, as well as from horses (n=10) with clinical signs of strangles and positive results from cultivation of *S. equi*. Microtiter wells (Costar) were coated overnight with 100 µl FNZN, SFSC1, EAG4B, SEC2.16 or SCLC1 at 10 µg per ml overnight in phosphate buffered saline (PBS). The plates were then blocked with 2% (w/v) BSA for 1 hour at 37°C. After washing, horse sera were added to wells at a 20-fold dilution, followed by 2-fold serial dilutions. The plates were washed after 1 hour incubation at 37°C. Detection of antibody binding was performed after washing, with antibodies, diluted 1000x, against horse IgG, raised in rabbits and conjugated with HRP (Sigma Chemical Co). Development of a colorimetric reaction was achieved with OPD tablets (Dako, Denmark). Absorbance was determined spectrophotometrically at 492 nm. The log<sub>10</sub> dilution of sera, which was required to give an absorbance at a cut-off value of 1.0, was calculated for each individual serum

sample. Mean values of the log<sub>10</sub> dilutions, with standard errors, are shown. For comparing normal vs. strangles sera against FNZN, SFSC1, EAG4B, SEC2.16 and SCLC1, the p-values are <0.001, 0.02, <0.0001, 0.02, and <0.0001, respectively.

In Figure 15, the results from a comparison of serum antibody titers from horses with or without strangles are shown. The designation n denotes normal horses without and the designatin s denotes horses with strangles. The log dilution of sera required to give an absorbance at a cut off value of 1.0 was calculated for each individual serum sample. Mean values with standard errors are shown. For comparing normal vs. strangles sera against FNZN, SFSC1, EAG4B, SEC2.16, and SCLC1, the p-values are <0.001, 0.02, <0.0001, 0.02 and < 0.0001, respectively.

#### **Example 14. Determination of IgA antibody titers from, immunized horses**

Horses were divided into four groups (A-D) and were immunized as described in Example 12. Antigen-specific IgA in nasal wash samples from immunized horses were determined by an indirect ELISA. Monoclonal antibody K129-3E7 (2 µg/ml) against equine IgA was used to detect bound IgA in the samples followed by use of a goat anti-mouse immunoglobulin HRP conjugate (2 µg/ml). Coating concentrations of antigens were 4 µg/ml. Background values (from no-antigen plates) were subtracted. All samples were analyzed in triplicate. Antigen-specific IgA ELISA data is expressed relative to the total IgA ELISA data in order to correct for nasal secretion dilution during the nasal wash procedure. Total IgA in nasal wash samples was determined by a capture ELISA using two different monoclonal antibodies specific to equine IgA. One was used to coat wells and the other was biotinylated and was detected with the use of a streptavidin-HRP conjugate. The concentrations of monoclonal antibody and streptavidin-HRP were optimized so that none of these was limiting in the assay. Samples were analysed in triplicate. In Figure 16, the Absorbance values obtained for IgA against FNZN and EAG4B are shown.

In Figure 16, IgA antibodies in nasal washings of immunized horses are shown. Mean absorbance values (n=3) in ELISA from groups A-D are shown. The horses were divided into four groups; A) antigens + EtxB given both s.c. and i.n.; B) as group A but immunization only i.n.; C) as in group A but omitting EtxB; D) control group given only EtxB both i.n. and s.c. Light gray bars: samples from day 0 and dark gray bars from day 56.

**Example 15. Determination of kinetics for development of IgG in immunized horses.**

Horses were immunized as described in Example 12. On days 0, 14, 28 and 42, samples were taken on these days and on day 56 as well and analyzed as described elsewhere.

- 5 The horses were immunized with EAG4B, FNZN and SFSC1 with EtxB, by both intranasal and subcutaneous route.

The antibody titers against EAG4B found in these (three) horses are shown in Figure 17 as a function of time.

**Example 16. Immunogenicity of protein SEC**

- 10 Mice were immunized intranasally with SEC2.16, with (n=5) or without Matrix (n=5) using 20 µg per mouse of both SEC2.16 and Matrix. Serum samples were taken before and after 4 immunizations, with two-week intervals between. Pre-immune sera did not contain any detectable antibodies against SEC2.16. SEC2.16 without any adjuvant gave significant antibody responses in absence of Matrix. In presence of Matrix even better antibody titers  
15 were obtained as shown in Figure 19.

**Example 17. Comparison of immunization with different antigens followed by challenge infection**

- Mice were immunized with proteins FNZN+SFSC1+EAG4B with EtxB (n=8) or with EAG4B with EtxB (n=10) as described in Example 11. Another group was given EtxB  
20 alone as control (n=9). The mice were subjected to challenge with subsp. *equi* and their weight change was monitored. As expected, the control group lost more weight than the groups that were given antigen, which confirms the protective effect of vaccination. The infected mice were left for 14 days. Animals in the group, which was given FNZN+SFSC1+EAG4B with EtxB as adjuvant regained weight from day 10, when average  
25 weight loss was approx. 10%, whereas on day 14, average loss was 7%. On the other hand, mice vaccinated with EAG4B with EtxB as adjuvant, kept losing weight and on day 14 weight loss in this group was 12.5%. The difference in weight loss on day 14 implies that supplying EAG4B together with other antigens, such as SFSC1 and/or FNZN improves protection. However, the difference is not significant due to small sample size. (No data  
30 shown)

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## CLAIMS

- 5           1. An antigenic composition comprising at least one antigen, wherein said at least one antigen comprises at least part of a protein of *Streptococcus equi* subsp *equi*, and said at least part of said protein comprises at least one antigenic epitope or antigenic determinant of *Streptococcus equi*, and wherein said protein is comprised of EAG comprising an amino acid sequence according to SEQ ID NO: 1, or an analog thereof.
- 10
2. The antigenic composition of claim 1, which comprises at least one further antigen that comprises at least part of a fibronectin-binding protein of *Streptococcus equi*, said at least part of said protein comprising at least one antigenic epitope or
- 15           antigenic determinant of *Streptococcus equi*, and wherein said protein is selected from the group consisting of FNZ comprising an amino acid sequence according to SEQ ID NO: 2 and SFS comprising an amino acid sequence according to SEQ ID NO: 3, or an analog thereof.
3. The antigenic composition of claim 1 or 2, which comprises at least one further antigen that comprises at least part of a protein of *Streptococcus equi* and said at least part of said protein comprises an antigenic epitope or an antigenic determinant of *Streptococcus equi*, and wherein said protein is comprised of SEC comprising an amino acid sequence according to SEQ ID NO: 4, or an analog thereof.
- 20
4. The antigenic composition of claim 1, 2, or 3 which comprises an N-terminal fragment of a protein selected from the group consisting of EAG and FNZ.
- 25
5. The antigenic composition of claim 3, wherein said antigens are comprised of at least part of EAG, FNZ, SFS, and SEC, optionally, said at least part of EAG and FNZ being an N-terminal part thereof, said at least part of SFS being a C-terminal part of SFS, and said at least part of SEC being a collagen-binding part of SEC.
- 30

6. A vaccine composition for protecting non-human mammals against infection of *Streptococcus equi*, which comprises the antigenic composition of claim 1 as immunizing component and a pharmaceutically acceptable carrier.

5 7. The vaccine composition of claim 6, which comprises the antigenic composition of any one of claims 2-5 as immunizing component.

8. The vaccine composition of claim 6 or 7, which further comprises an adjuvant.

10 9. The vaccine composition of any one of claims 6-8, which is a vaccine that protects susceptible mammals, suitably horses, against strangles caused by *Streptococcus equi* subsp. *equi*.

15 10. The vaccine composition of claim 7, which is provided in a physiologically administrable form and suitably is administrable by subcutaneous or intranasal inoculation.

20 11. The vaccine composition of claim 9 or 10, which stimulates serum, mucosal and/or bronchial lavage antibody responses directed to *Streptococcus equi* antigens in mammals susceptible to *Streptococcus equi*, suitably horses.

12. A method for producing an antigen of an antigenic composition of any one of claims 1-5, which method comprises

25 (a) providing a DNA fragment encoding said antigen and introducing said fragment into an expression vector;

(b) introducing said vector, which contains said DNA fragment, into a compatible host cell;

(c) culturing said host cell provided in step (b) under conditions required for expression of the product encoded by said DNA fragment; and

30 (d) isolating the expressed product from the cultured host cell, and, optionally,

(e) purifying the isolated product from step (d) by affinity chromatography or other chromatographic methods known in the art.

13. A method for preparation of a vaccine according to any one of claims 6-11, which vaccine contains as immunizing component, an antigenic composition of any one of claims 1-5, said method comprising mixing said antigenic composition and a pharmaceutically acceptable carrier.

5

14. Use of an antigenic composition of any one of claims 1-5 in the preparation of a vaccine protecting against *S. equi* infection inclusive of strangles caused by subsp. *equi* infection in horses.

10

15. A method for the production of an antiserum, said method comprising administering an antigenic preparation of any one of claims 1-5 to an animal host to produce antibodies in said animal host and recovering antiserum containing said antibodies produced in said animal host.

15

16. A method of prophylactic or therapeutic treatment of *S. equi* infection in non-human mammals, suitably horses, comprising administering to said mammal an immunologically effective amount of a vaccine of any one of claim 6-11 or an antiserum produced according to claim 15.

20

17. A method for protecting horses against *Streptococcus equi* infection, which comprises inoculating a horse subcutaneously or intranasally with a vaccine of any one of claims 6-11 to induce an immune response against *Streptococcus equi* in said horse.

25

18. The method of claim 17, wherein an immune response in the form of IgG and/or IgA and/or IgM antibodies in the nasopharyngeal mucus is induced in said horse.

19. Monoclonal antibodies against antigen(s) of the composition of any of claims 1-4 to be used prophylactically or therapeutically against strangles.

30

20. The antigenic composition of any one of claims 1-5, which comprises at least part of a protein designated Scl and comprising an amino acid sequence according to SEQ ID. NO:23 or a fragment thereof, suitably a fragment designated SCL C1 and

comprising an amino acid sequence according to SEQ. ID. NO:27, or an analog thereof.

Fig 1

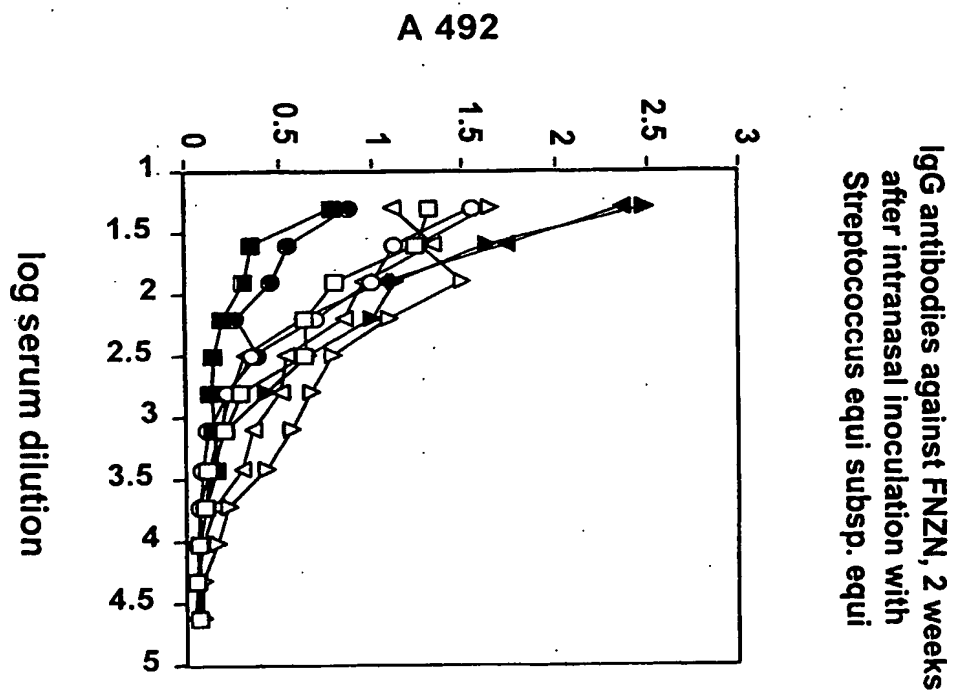
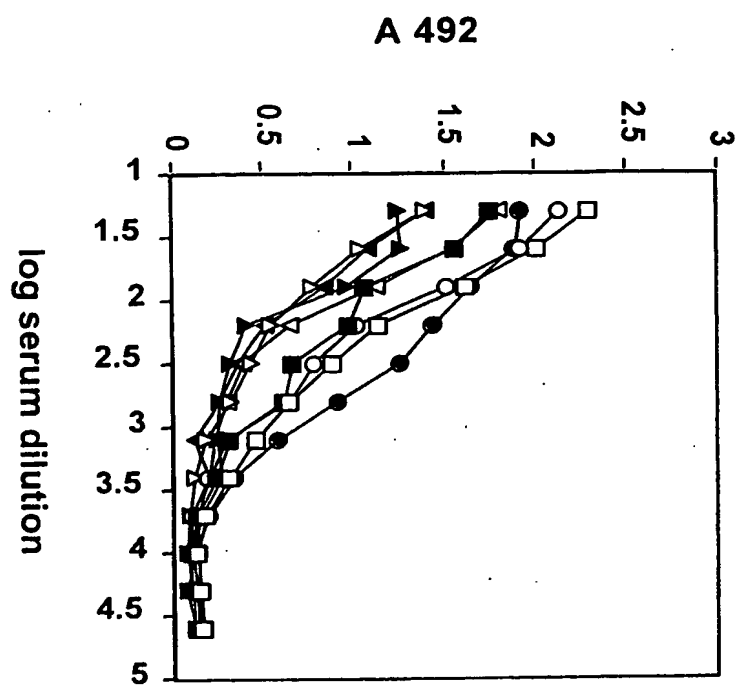


Fig 2



IgG antibodies against SFSC1, 2 weeks  
after intranasal inoculation with  
*Streptococcus equi* subsp. *equi*

Fig 3

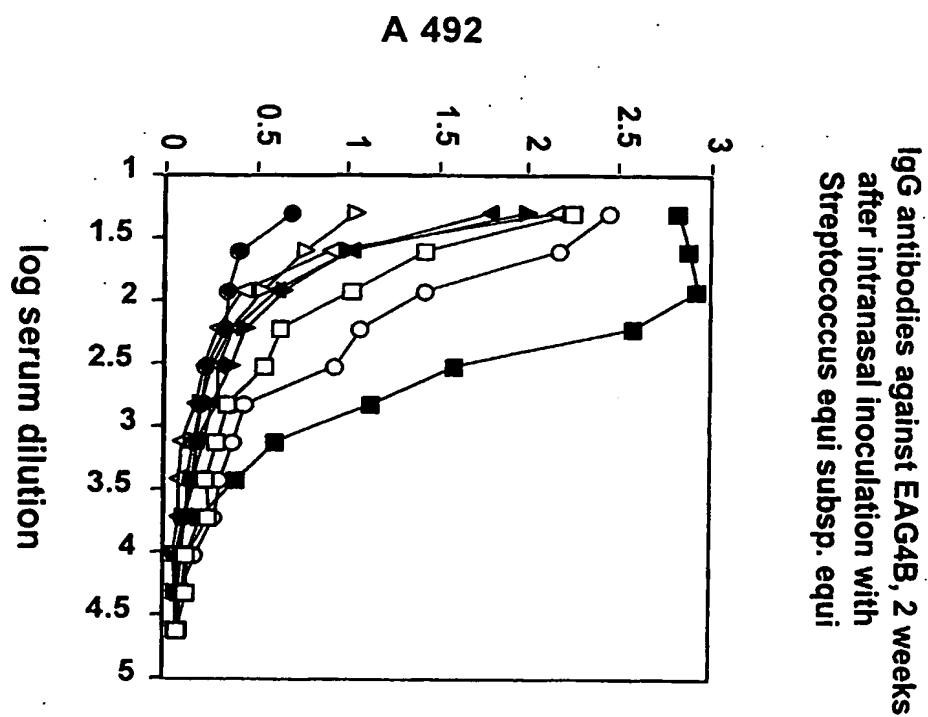
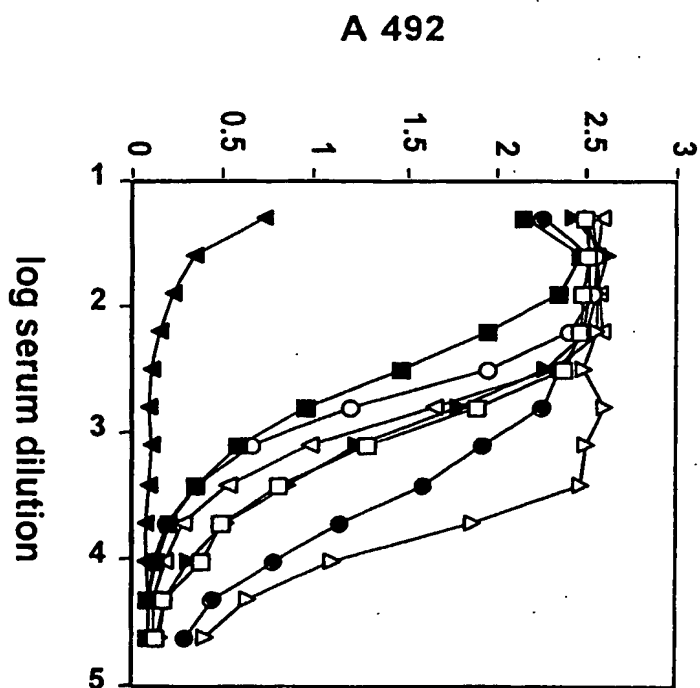


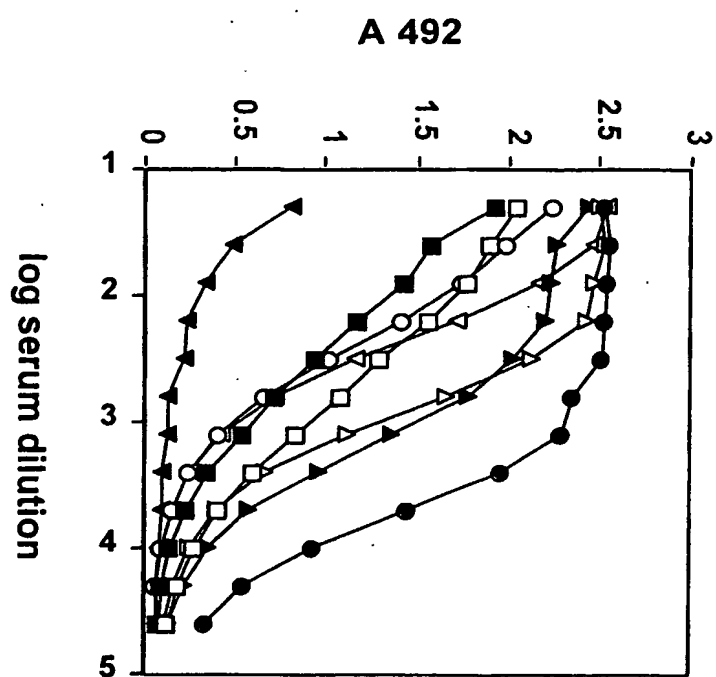
Fig 4



IgG antibodies against FNZN  
after subcutaneous immunization  
with FNZN, SFSC1 o EAG4B

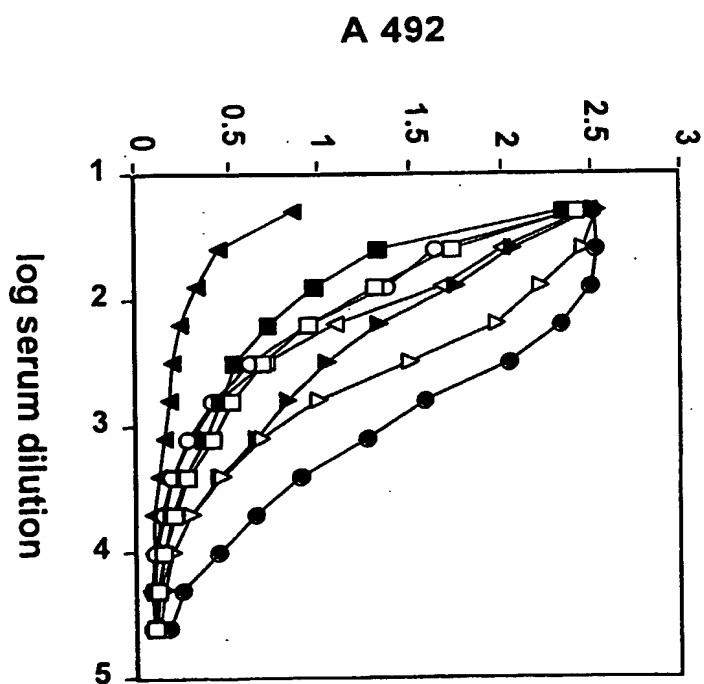


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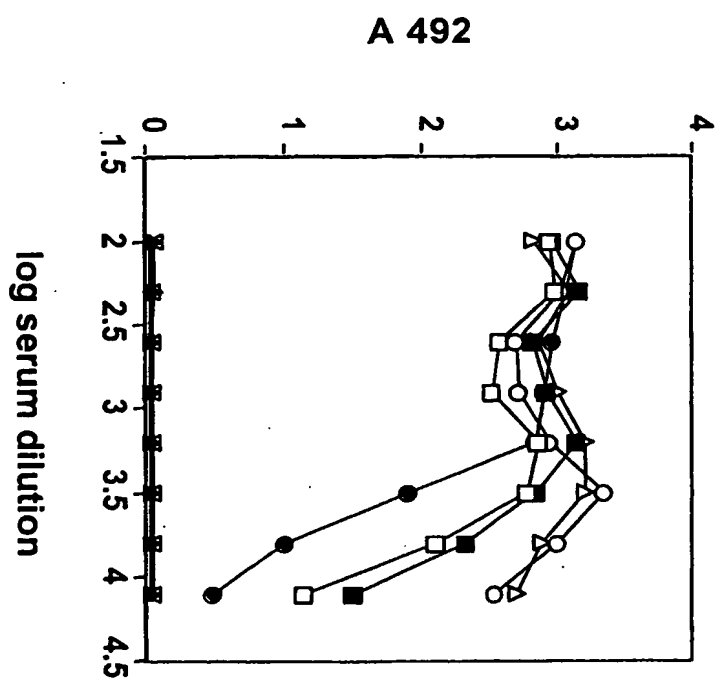
IgG antibodies against SFSC1  
after subcutaneous immunization  
with FNZN, SFSC1 o EAG4B

Fig 6



IgG antibodies against EAG4B  
after subcutaneous immunization  
with FNZN, SFSC1 o EAG4B

Fig 7



IgG antibodies against SEC 2.16 after  
subcutaneous immunization with SEC 2.16

Fig 8

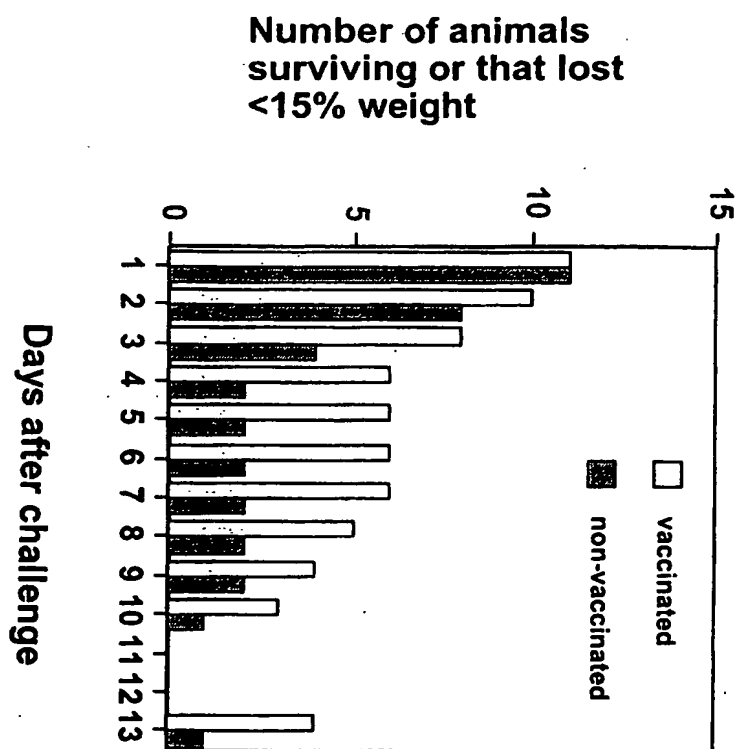


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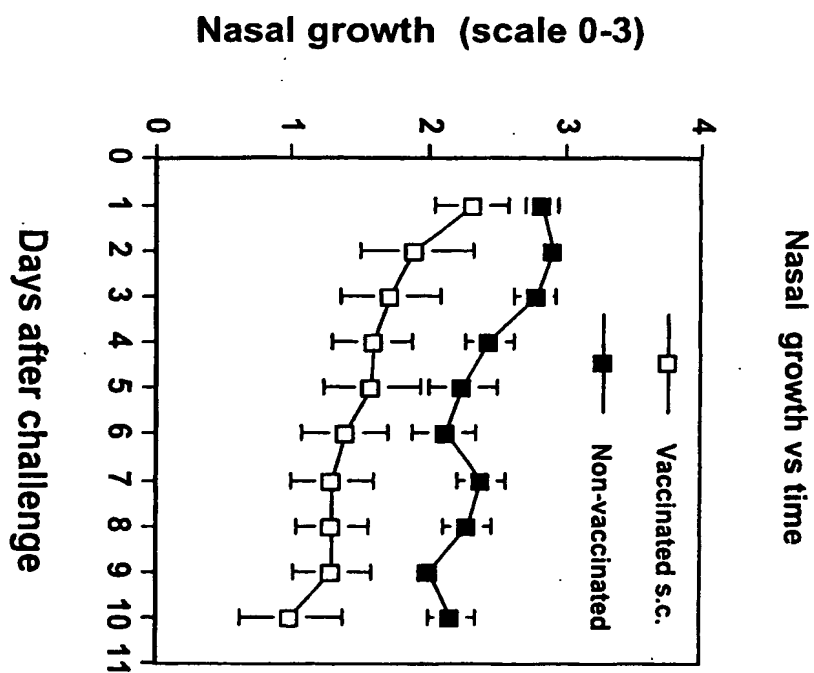


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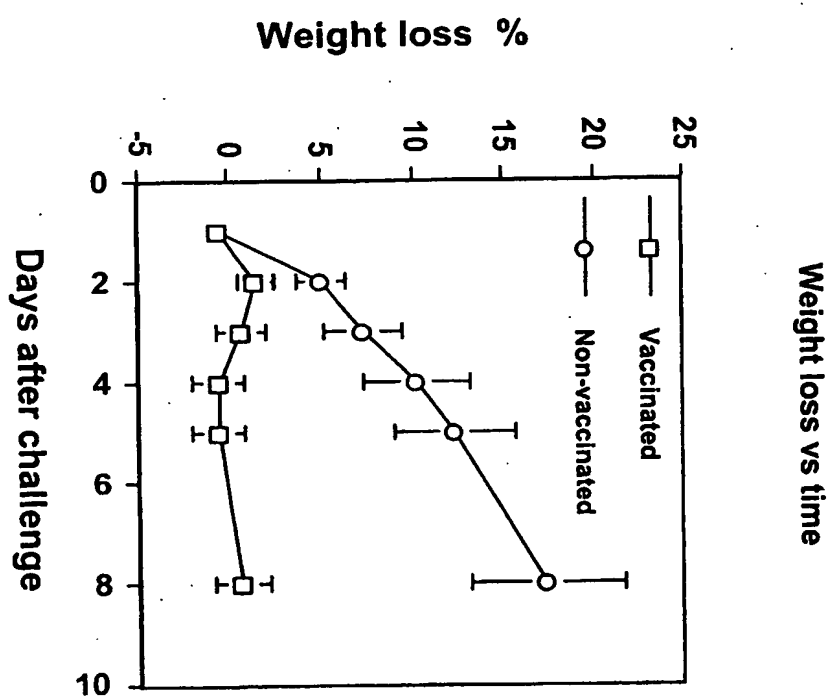


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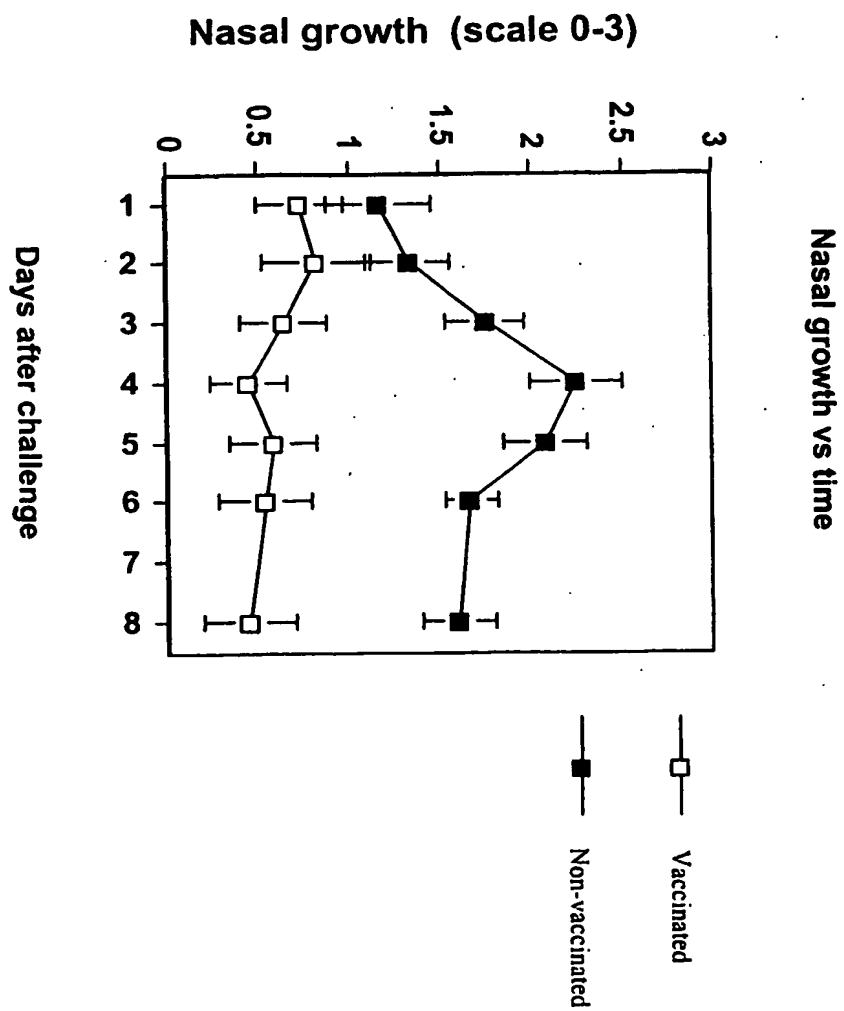


Fig 12

BAL/NW IgA antibodies against  
SFSC1 after intranasal immunization  
with FNZN, SFSC1 and EAG4B

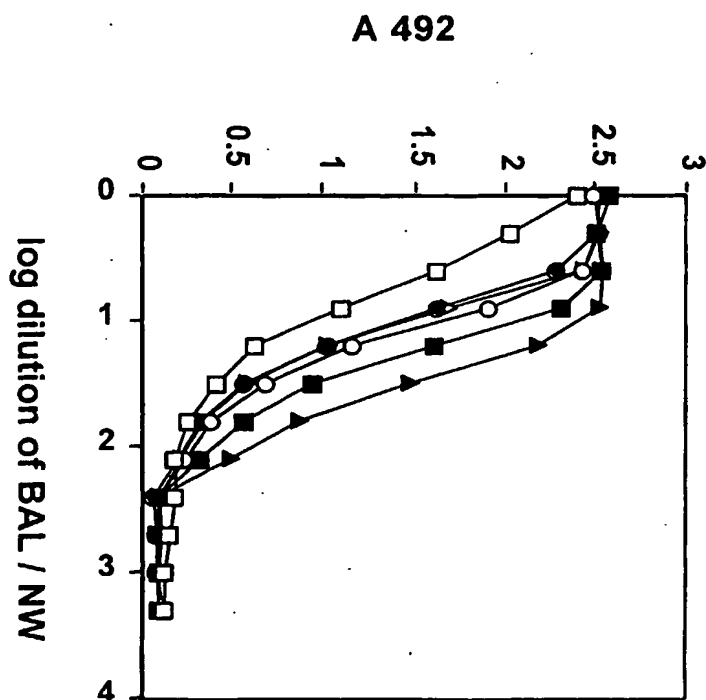




Fig 13

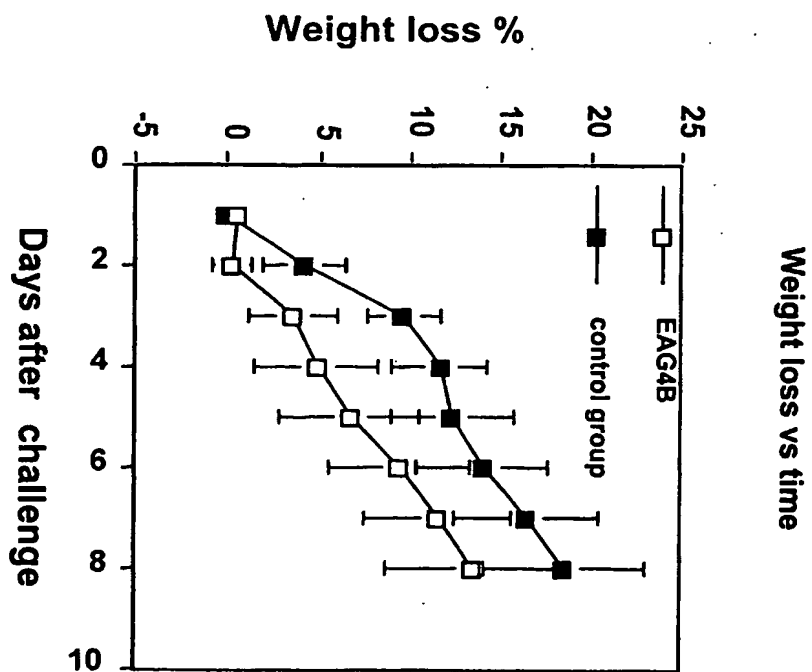


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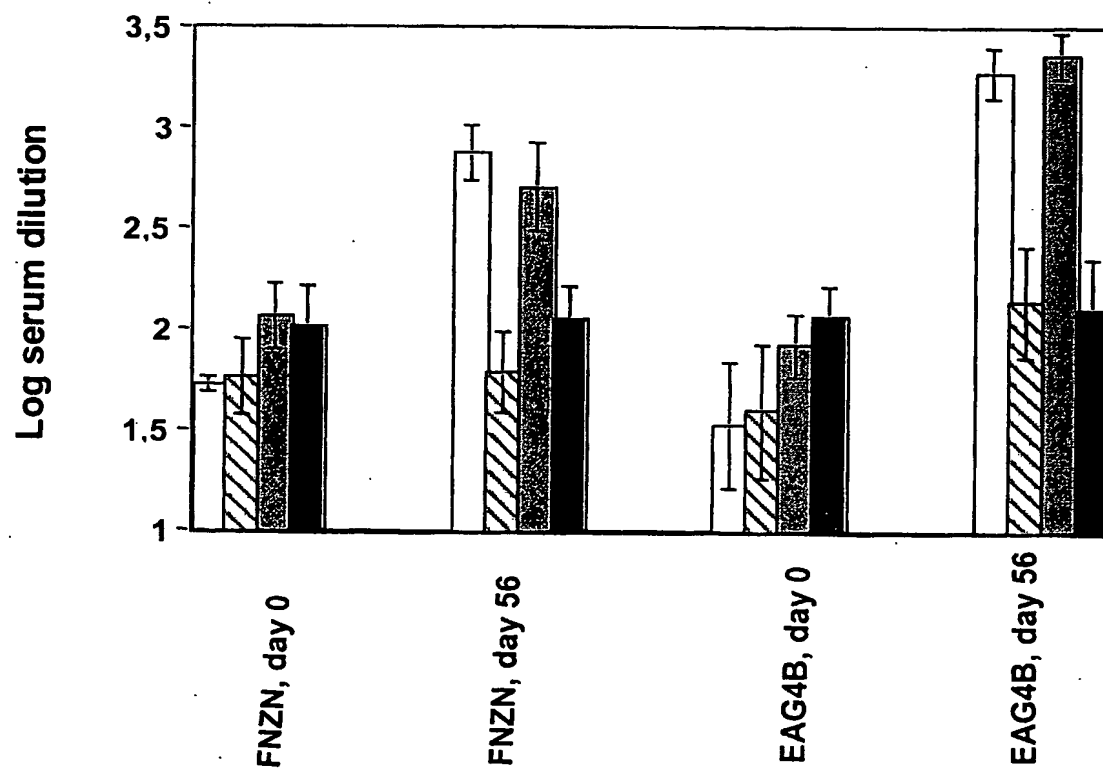


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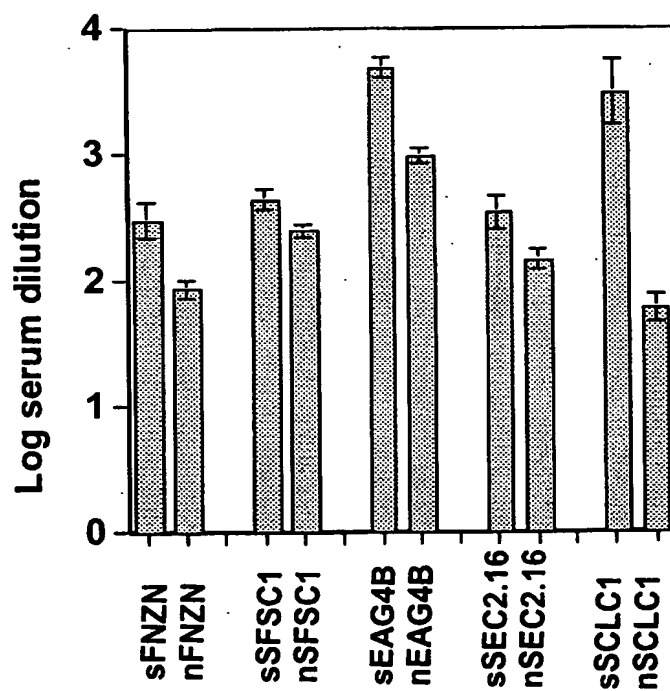


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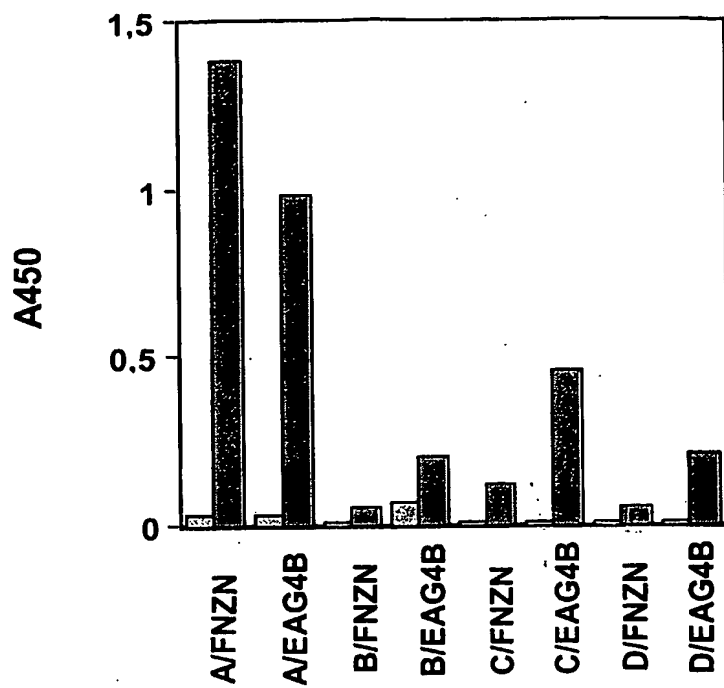


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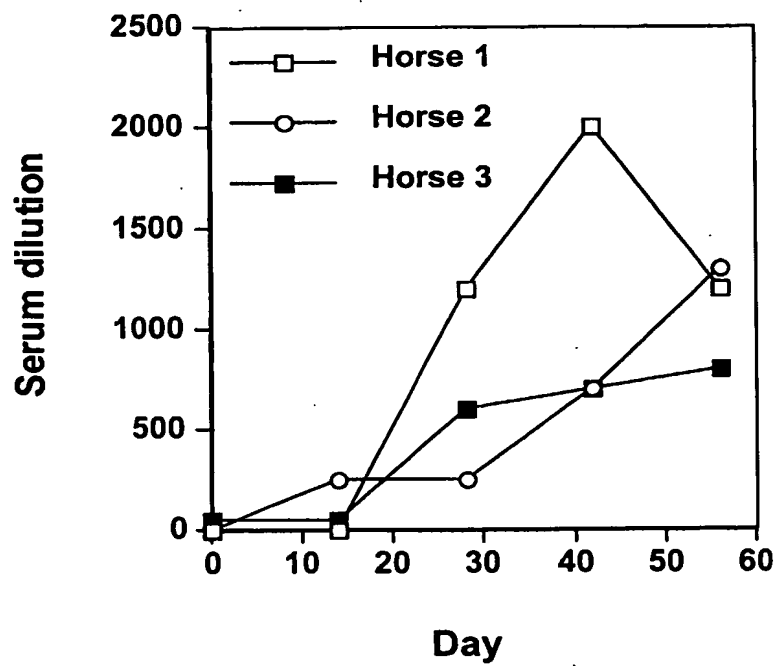


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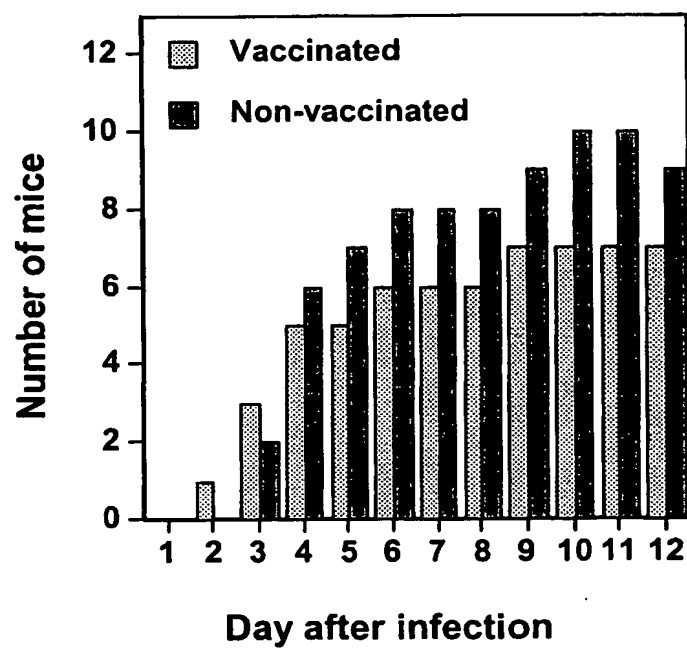
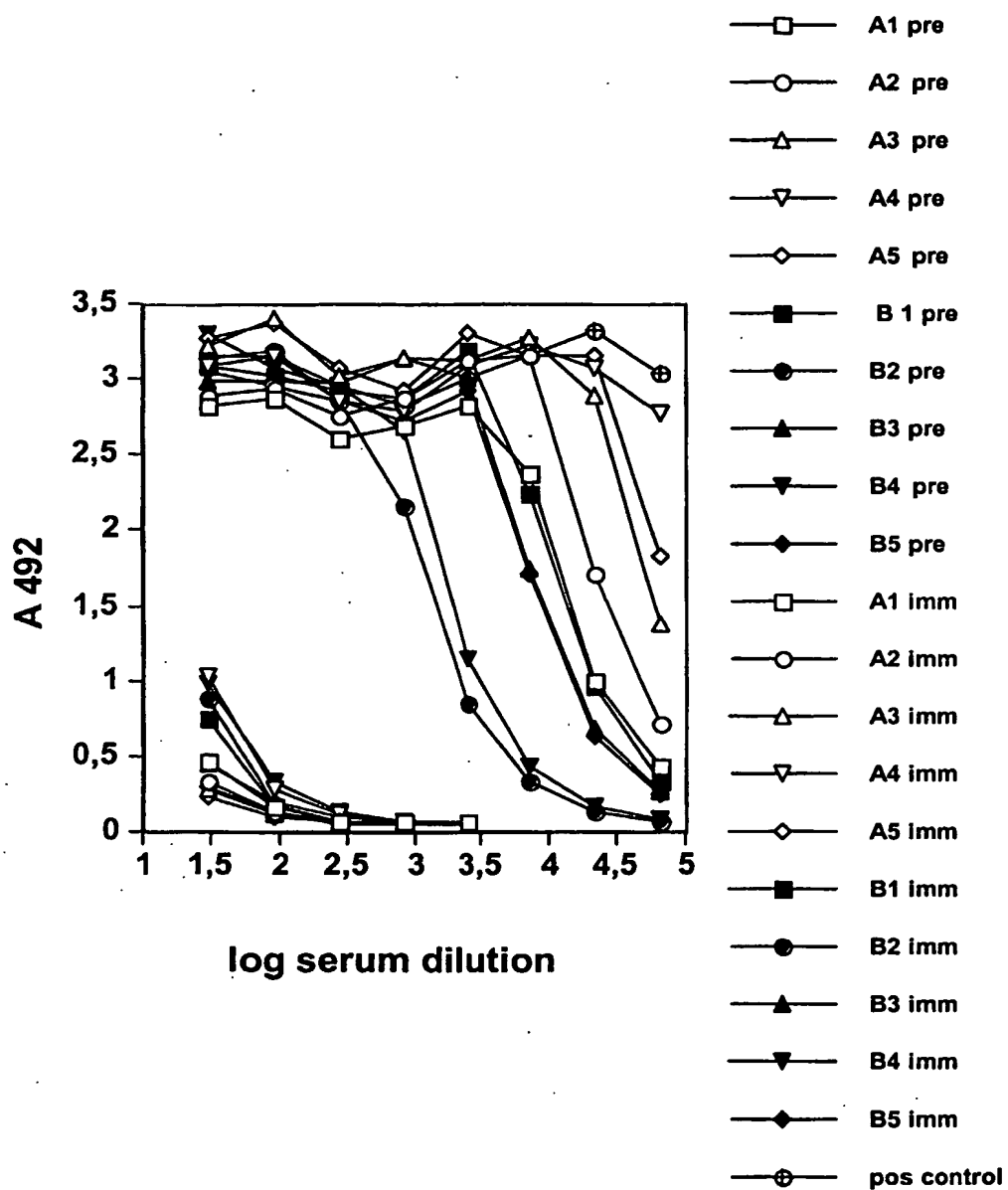


Figure 19



110087400US.ST25  
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<120> Immunization of non-human mammals against Streptococcus equi

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<150> US 60/417,660

<151> 2002-10-11

<160> 27

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Gln Leu Ala Leu Val Ala Ala Ala Ala Lys Val Ala Asp Ala Ala Ser  
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 Lys Asp Thr Gly Gly Tyr Asn Leu Asn Asp Met Glu Lys Lys Ala Leu  
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 Asp Phe Leu Ile Ser Lys Gly Glu Asp Ser Lys Leu Lys Ser Glu Gln  
 195 200 205  
 Ser Asn Tyr Ser Leu Asp Ile Tyr Val Tyr Gln Ser Gly Gly His Asp  
 210 215 220  
 His Met Lys Asp Tyr Gln Asn Leu Leu Gly Ser Thr Leu Ile Pro Lys  
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 245 250 255  
 Gly Leu Ser Gly Leu Glu Gly Gly Ser Ser Gly Ser Gln Glu Thr Asn  
 260 265 270  
 Glu Asp Gly Lys Lys Gly Leu Ile Gly Phe His Gly Gly Leu Ser Gly  
 275 280 285  
 Ser Glu Gly Lys Arg Asp Pro Leu Pro Gly Leu Lys Gly Glu Ala Gly  
 290 295 300  
 Ala Pro Asp Thr Pro Gln Lys Pro Asn Asp Pro Leu Gln Gly Leu Glu  
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agatgctg	ct	tcagttg	atc	aagtgaat	gc	agccatta	at	gatgctc	ata	cagctatt	gc	420
ggacatt	taca	ggagcag	cct	tgttggag	gc	taaagaag	ct	gctatca	atg	aactaaag	ca	480
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&lt;213&gt; Streptococcus equi

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Ser Ser Leu Arg Asp Gly Glu Arg Thr Thr Val Lys Val Ala Phe Asp  
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 Lys Ala Val Phe Thr Phe Asn Gln Asn Ile Glu Thr Met His Asp Val  
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 Asp Gln Lys Glu Phe Ala Asn Asn Ser Lys Ile Trp Tyr Gln Ile Leu  
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 Lys Leu Asp Lys Tyr Asp Ser Gln Asn Gln Lys Ile Thr Tyr Ser Val  
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 Lys Glu Val Met Val Pro Val Gly Tyr Gln Ser Gln Val Glu Gly Asp  
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 Ser Gly Val Gly Phe Thr Ile Thr Asn Thr Tyr Thr Pro Glu Val Ile  
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 Pro Val Tyr Gln Ala Gly Lys Lys Ile Ser Tyr Ser Ile Glu Glu Lys  
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&lt;212&gt; DNA

&lt;213&gt; Streptococcus equi

&lt;400&gt; 21

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&lt;212&gt; PRT

&lt;213&gt; Streptococcus equi

&lt;400&gt; 22

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 Pro Thr Ser Gly Asn Val Tyr Ile Gln Gly Phe Asn Lys Thr Ile Pro  
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 Lys Ala Val Phe Thr Phe Asn Gln Asn Ile Glu Thr Met His Asp Val  
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 130 135 140  
 Phe Tyr Tyr Lys Thr Gly Asp Met Gln Pro Asn Asp Thr Glu Arg Val  
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Ala Ser Glu Gln Ser Ser Asn Pro Lys Ala Pro Ala Pro Lys Ser Ala  
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&lt;211&gt; 909

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 85 90 95

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Pro Arg Gly Glu Gln Gly Pro Ala Gly Gln Ala Gly Glu Arg Gly Pro  
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225 230 235

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 2003/001587

## A. CLASSIFICATION OF SUBJECT MATTER

IPC7: A61K 39/09, C07K 14/315, C07K 16/12

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C07K, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-INTERNAL, WPI-DATA, PAJ, BIOSIS, MEDLINE, CHEM. ABS DATA

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	FEMS Microbiology Letters, Volume 222, 2003, Jonas Lannergård et al: "CNE, a collagen-binding protein of Streptococcus equi", page 69 - page 74, abstract; page 70, column 2, paragraph 1; page 73, column 2, paragraph 2	12,19
P,Y	--	3,5,7-19
X	Hans Lindmark, "Acta Universitatis Agriculturae Sueciae, AGRARIA 139, Characterization of Adhesive Extracellular Proteins from Streptococcus equi", 1999, Doctoral thesis Swedish University of Agricultural Sciences, Uppsala, page 1 - page 49, abstract; page 25 - page 35	1-2,4-19
Y	--	2-5,7-19

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

\* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

12 January 2004

Date of mailing of the international search report

20 -01- 2004

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Terese Persson/Eö  
Telephone No. +46 8 782 25 00

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 2003/001587

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Hans Lindmark et al, "Acta Universitatis Agruculturae Sueciae, AGRARIA 139, Characterization of Adhesive Extracellular Proteins from Streptococcus equi", 1999, The NH2-terminal half of protein FNZ binds fibronectin and is secreted in S. equi subsp. equi but not in subsp. zooepidemicus, Swedish University of Agricultural Sciences, Uppsala, page 1 - page 13, abstract; page 93, column 2, paragraph 2; page 96	1-2,4-19
Y	--	2-5,7-19
X	Research in Veterinary Science, Volume 66, 1999, Hans Lindmark et al: "Pulsed-field gel electrophoresis and distribution of the genes zag and fnz in isolates of Streptococcus equi", page 93 - page 99, abstract; page 93, column 2, paragraph 2; page 96	1-2,4-19
Y	--	2,5,7-19
X	Infection and Immunity, Volume 67, no. 5, May 1999, Hans Lindmark et al: "SFS, a Novel Fibronectin-Binding Protein from Streptococcus equi, Inhibits the Binding between Fibronectin and Collagen", page 2383 - page 2388, abstract; page 2384, column 1, paragraph 5	12,19
Y	--	2,5,7-19
X	Infection and Immunity, Volume 69, no. 5, May 2001, Hans Lindmark et al: "Comparison of the Fibronectin-Binding Protein FNE from Streptococcus equi Subspecies equi with FNZ from S. equi Subspecies zooepidemicus Reveals a Major and Conserved Difference", page 3159 - page 3163, abstract; page 3160, column 2, paragraph 3	12,19
Y	--	2,4,5,7-19



# INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 2003/001587

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 0037496 A1 (GUSS, BENGT ET AL), 29 June 2000 (29.06.2000), claims	12,19
Y	--	2,5,7-19
X	WO 9507296 A1 (GUSS, BENGT ET AL), 16 March 1995 (16.03.1995), abstract; claims	1,6,8-9,11
Y	-- -----	2,3,7-19

# INTERNATIONAL SEARCH REPORT

International application No.  
**PCT/SE03/01587**

## Box No. II Observations where certain claims were found unsearchable (Continuation of Item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: **16-18**  
because they relate to subject matter not required to be searched by this Authority, namely:  
**see next sheet\***
2. ☒ Claims Nos.: **20 (partially)**  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
**see next sheet\*\***
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

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Claims 16-18 relate to methods of treatment of the human or animal body by surgery or by therapy or diagnostic methods practised on the human or animal body (PCT Rule 39.1(iv)). Nevertheless, a search has been executed for these claims. The search has been based on the alleged effects of the compounds or compositions.

★★

Present claim 20 relates to an extremely large number of possible protein fragments due to the expression "fragment thereof". Support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the protein fragments. In the present case, the claim so lacks support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible.

Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts related to the protein Sc1C and the fragment Sc1C1, as well as general aspects thereof.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established will not be, the subject of an international preliminary examination (Rule 66.1(e) PCT). This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

01/12/2003

International application No.  
**PCT/SE 2003/001587**

WO	0037496	A1	29/06/2000	AU	3094700	A	12/07/2000
				CA	2355831	A	29/06/2000
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				AU	7628194	A	27/03/1995
				CA	2176230	A	16/03/1995
				DE	69425149	D,T	15/03/2001
				EP	0724596	A,B	07/08/1996
				ES	2149884	T	16/11/2000
				SE	9302855	D	00/00/0000
				US	6100055	A	08/08/2000

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